



PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/74, 15/60, 9/88, 1/21	A2	(11) International Publication Number: WO 95/03417 (43) International Publication Date: 2 February 1995 (02.02.95)
---	----	---

(21) International Application Number: PCT/US94/08267

(22) International Filing Date: 22 July 1994 (22.07.94)

(30) Priority Data:
08/095,734 22 July 1993 (22.07.93) US

(71) Applicant: WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH [US/US]; Nine Cambridge Center, Cambridge, MA 02142 (US).

(72) Inventors: ALDOVINI, Anna; 26 Highland Street, Weston, MA 02193 (US). YOUNG, Richard, A.; 26 Highland Street, Weston, MA 02193 (US).

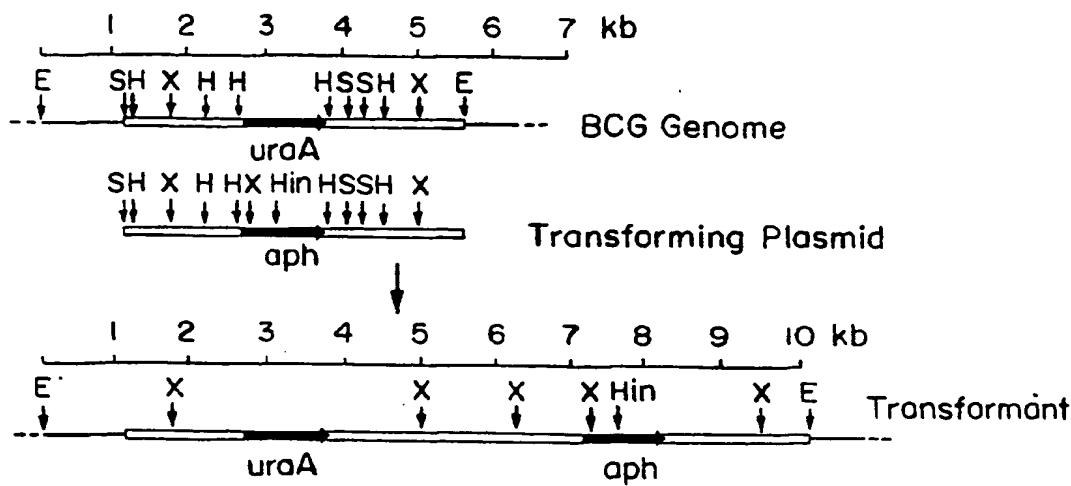
(74) Agents: GRANAHAN, Patricia et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US).

(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: HOMOLOGOUSLY RECOMBINANT SLOW GROWING MYCOBACTERIA AND USES THEREFOR



(57) Abstract

A method of transforming slow-growing mycobacteria, such as *M. bovis* BCG, *M. leprae*, *M. tuberculosis*, *M. avium*, *M. intracellulare* and *M. africanum*; a method of manipulating genomic DNA of slow-growing mycobacteria through homologous recombination; a method of producing homologously recombinant (HR) slow-growing mycobacteria in which heterologous DNA is integrated into the genomic DNA at a homologous locus; homologously recombinant (HR) slow-growing mycobacteria having heterologous DNA integrated into their genomic DNA at a homologous locus; and mycobacteria DNA useful as a genetic marker.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

HOMOLOGOUSLY RECOMBINANT SLOW GROWING
MYCOBACTERIA AND USES THEREFOR

Description

Background of the Invention

5 The World Health Organization estimates that one in three human beings is believed to be infected with Mycobacterium tuberculosis (Styblo, K., Reviews of Infectious Diseases. Vol. II, Suppl. 2, March-April, 1989; Bloom and Murray, Science 257:1055-1067, 1992). Over the past
10 decade, there has been a recent resurgence in the incidence of tuberculosis in developed countries that has coincided with the AIDS epidemic (Snider and Roper, N. England J. Med. 326:703-705 (1992)). Because of their impact as major human pathogens and as a result of their
15 profound immunostimulatory properties, mycobacteria have long been intensively studied. In the early 1900s, an attenuated mycobacterium, Mycobacterium (M.) bovis Bacille Calmette-Guerin (M. bovis BCG or BCG), was isolated for use as a vaccine against tuberculosis (Calmette *et al.*
20 Acad. Natl. Med. (Paris), 91:787-796, 1924; reviewed in Collins, F.M., Bacterial Vaccines (R. Germanier, ed.), Academic Press, pp. 373-418, 1984). Although the efficacy of this vaccine against tuberculosis varied considerably in different trials, and the reasons for its variable
25 efficacy have yet to be resolved, BCG is among the most widely used human vaccines (Luelmo, F., Am. Rev. Respir. Dis. 125:70-72, 1982; Fine, P.E.M., Reviews of Infectious Diseases II (supp. 2), 5353-5359, 1989).

30 The recent application of molecular biological technology to the study of mycobacteria has led to the identification of many of the major antigens that are targets of

-2-

the immune response to infection by mycobacteria (Kaufmann, S.H.E., Immunol. Today 11:129-136, 1990; Young, R.A., Ann. Rev. Immunol. 8:401-420, 1990; Young et al., Academic Press Ltd., London, pp. 1-35, 1990; Young et al., Mol. Microbiol. 6:133-145, 1992)) and to an improved understanding of the molecular mechanisms involved in resistance to antimycobacterial antibiotics (Zhang et al., Nature 358:591-593, 1992; Telenti et al., Lancet 341:647-650, 1993). The development of tools that permit molecular genetic manipulation of mycobacteria has also allowed the construction of recombinant BCG vaccine vehicles (Snapper et al., Proc. Natl. Acad. Sci. USA 85:6987-6991, 1988; Husson et al., J. Bacteriol. 172:519-524, 1990; Martin et al., B. Nature 345:739-743, 1990; Snapper et al., Mol. Microbiol. 4:1911-1919, 1990; Aldovini and Young, Nature 351:479-482, 1991; Jacobs et al., Methods Enzymol. 204:537-555, 1991; Lee et al., Proc. Natl. Acad. Sci. USA 88:3111-3115, 1991; Stover et al., Nature 351:456-460, 1991; Winter et al., Gene 109:47-54, 1991; Donnelly-Wu et al., Mol. Microbiol. 7:407-417, 1993)). Genome mapping and sequencing projects are providing valuable information about the M. tuberculosis and M. leprae genomes that will facilitate further study of the biology of these pathogens (Eiglmeier et al., Mol. Microbiol., in press, 1993; Young and Cole, J. Bacteriol. 175:1-6, 1993).

Despite these advances, there are two serious limitations to our ability to manipulate these organisms genetically. First, very few mycobacterial genes that can be used as genetic markers have been isolated (Donnelly-Wu et

al., Mol. Microbiol. 7:407-417, 1993)). In addition, investigators have failed to obtain homologous recombination in slow growing mycobacteria, such as M. tuberculosis and M. bovis BCG (Kalpana et al., Proc. Natl. Acad. Sci. USA 88:5433-5447, 1991; Young and Cole, J. Bacteriol. 175:1-6, 1993)), although homologous recombination has been accomplished in the fast growing Mycobacterium smegmatis (Husson et al., J. Bacteriol. 172: 519-524, 1990)).

10 Summary of the Invention

Described herein is a method of transforming slow-growing mycobacteria, such as M. bovis BCG, M. leprae, M. tuberculosis M. avium, M. intracellulare and M. africanum; a method of manipulating genomic DNA of slow-growing mycobacteria through homologous recombination; a method of producing homologously recombinant (HR) slow-growing mycobacteria in which heterologous DNA is integrated into the genomic DNA at a homologous locus; homologously recombinant (HR) slow-growing mycobacteria having heterologous DNA integrated into their genomic DNA at a homologous locus; and mycobacterial DNA useful as a genetic marker.

Applicants have succeeded in introducing heterologous DNA into (i.e., transforming) slow-growing mycobacteria through the use of electroporation in water (rather than in buffer). In the present method of transforming slow-growing mycobacteria, heterologous DNA (such as linear DNA or plasmid DNA) and slow-growing mycobacteria (e.g., M. bovis BCG, M. leprae, M. tuberculosis M. avium, M. intracellulare and M. africanum) are combined and the

- 4 -

resulting combination is subjected to electroporation at an appropriate potential and capacitance for sufficient time for the heterologous DNA to enter the slow growing mycobacteria, resulting in the production of transformed 5 mycobacteria containing the heterologous DNA. In one embodiment, heterologous DNA and M. bovis BCG are combined and subjected to electroporation in water. In a particular embodiment, the M. bovis BCG-heterologous DNA combination is subjected to electroporation in water at settings 10 of approximately 2.5kV potential and approximately 25 μ F capacitance. Optionally, prior to harvest, cells to be transformed are exposed to glycine (such as by adding 1-2% glycine to culture medium in which the slow-grow mycobacteria are growing) in order to enhance or improve transformation 15 efficiencies. In one embodiment, 1.5% glycine is added to the culture medium 24 hours prior to harvesting of the cells, which are then combined with heterologous DNA to be introduced into the slow-growing mycobacteria. The resulting combination is subjected to electroporation, 20 preferably in water, as described above.

In a further embodiment of the method of transforming slow growing mycobacteria, cultures of the cells are maintained in (continuously propagated in) mid-log growth, in order to increase the fraction of cells which are 25 undergoing DNA synthesis (and which, thus, are competent to take up heterologous DNA). Cultures of cells maintained in log-phase growth are subjected to electroporation, preferably in water and, as a result, are transformed with the heterologous DNA. As described above, 30 efficiency of transformation can be increased by exposing

the slow-growing mycobacteria to glycine prior to electroporation. Thus, in this embodiment, slow-growing mycobacteria in log-phase growth are combined with heterologous DNA (e.g., plasmid DNA, linearized DNA) to be introduced 5 into the slow-growing mycobacteria. The resulting combination is subjected to electroporation (preferably in water), under conditions (potential and capacitance settings and sufficient time) appropriate for transformation of the cells. Optionally, prior to electroporation, the 10 log-phase cells are exposed to glycine (e.g., approximately 1-2% glycine added to culture medium) in order to enhance transformation efficiency.

Heterologous DNA introduced into slow-growing mycobacteria is DNA from any source other than the recipient 15 mycobacterium. It can be homologous to DNA present in the recipient mycobacterial genomic DNA, nonhomologous or both. DNA which is homologous to mycobacterial genomic DNA is introduced into the genomic DNA by homologous recombination or integration. Alternatively, the heterologous DNA introduced by the present method can be nonhomologous and, thus, enter mycobacterial genomic DNA by random integration events or remain extrachromosomal (unintegrated) after it enters the mycobacterium. In addition, in one embodiment of the present method, non- 20 homologous DNA linked to or inserted within DNA homologous to genomic DNA of the recipient mycobacterium is introduced into genomic DNA of the recipient mycobacterium as a result of homologous recombination which occurs between genomic DNA and the homologous DNA to which the nonhomologous DNA is linked (or in which it is inserted). For 25 30

example, as described herein, a mycobacterial gene which encodes a genetic marker has been identified and isolated and used to target homologous integration of heterologous DNA (DNA homologous to genomic DNA of the mycobacterial recipient, alone or in conjunction with DNA not homologous to genomic DNA of recipient mycobacteria) into genomic DNA of a slow-growing mycobacterium. Specifically, the M. bovis BCG gene encoding orotidine-5- monophosphate decarboxylase (OMP DCASE) (uraA) has been isolated, as has DNA flanking OMP DCASE. The OMP DCASE gene and the flanking DNA have been sequenced. The mycobacterial DNA containing the uraA locus, modified to contain heterologous DNA (a selectable marker gene) has been used to carry out integration of the heterologous DNA (the mycobacterial DNA and the selectable marker gene) into mycobacterial genomic DNA, resulting in production of homologously recombinant mycobacteria containing the heterologous DNA of a homologous locus. Specifically, M. bovis BCG DNA containing the uraA locus and flanking sequences was modified to replace the OMP DCASE coding sequence with the Kan^r selectable marker gene (aph). The resulting construct, which included approximately 1.5 kb uraA flanking sequences on each side of the selectable marker gene, was transformed into M. bovis BCG, using the method described above. M. bovis BCG cultures in mid-log growth were subjected to electroporation in water, resulting in transformation of cells with the construct. Transformants were selected for further study, which showed that all transformants assed contained vector DNA integrated into the genome and that in some of the transformants, the transforming DNA

had integrated at the homologous genomic locus. Thus, heterologous DNA of interest has been introduced into genomic DNA of slow-growing mycobacteria through homologous recombination, to produce homologously recombinant 5 slow-growing mycobacteria in which the heterologous DNA is integrated into the homologous genomic locus (a genomic locus homologous to at least a portion of the heterologous DNA).

Heterologous DNA which includes DNA homologous to 10 genomic DNA of the recipient mycobacterium (homologous DNA) and DNA which is not homologous to genomic DNA of the recipient mycobacterium (nonhomologous DNA) can be introduced into (transformed into) slow growing mycobacterium by the present method for several purposes. As described 15 herein, heterologous nonhomologous DNA encoding a product to be expressed by the resulting homologously recombinant slow-growing mycobacterium has been introduced into mycobacterial genomic DNA at a locus homologous with additional sequences to which the nonhomologous DNA is linked. In 20 this embodiment, the DNA construct transformed into recipient slow-growing mycobacteria comprises homologous DNA, which directs or targets introduction of the heterologous DNA into the homologous locus of the mycobacterial genome, and nonhomologous DNA, which is expressed in transformed 25 homologously recombinant mycobacteria. In this embodiment, the nonhomologous DNA is introduced into mycobacterial genomic DNA in such a manner that it is added to the genomic DNA or replaces genomic DNA. In a second embodiment, heterologous DNA integrated into genomic DNA is not 30 expressed in the recipient cells. In this embodiment, the

DNA construct includes homologous DNA for targeting into a homologous genomic locus and DNA which acts to knock out (inactivate) or activate a resident mycobacterial gene. In the case of inactivation, the mycobacterial gene is 5 "knocked out", in the sense that it is rendered inactive by addition of DNA whose presence interferes with its ability to function, by removal or replacement of sequences necessary for it to be functional or by its complete removal from the mycobacterial genome. In the case 10 of activation, the heterologous DNA integrated into the genomic DNA turns on or enhances expression of a mycobacterial gene, such as by introducing a heterologous promoter which controls the mycobacterial gene expression. In the embodiment in which heterologous DNA affects expression 15 of an endogenous mycobacterial gene, the homologous DNA can serve both functions (i.e., the targeting and inactivation/activating functions); if that is the case, the DNA construct includes only homologous DNA. Alternatively, the DNA construct can include homologous DNA (for 20 targeting purposes) and nonhomologous DNA (for altering function of the mycobacterial gene).

Homologously recombinant slow-growing mycobacteria of the present invention are useful, for example, as vehicles in which proteins encoded by the heterologous nonhomologous DNA are expressed. They are useful as vaccines, 25 which express a polypeptide or a protein of interest (or more than one polypeptide or protein), such as an antigen or antigens of one or more pathogens against which protection is desired (e.g., to prevent or treat a disease or 30 condition caused by the pathogen). Pathogens of interest

- 9 -

include viruses, retroviruses, bacteria, mycobacteria, other microorganisms, organisms or substances (e.g., toxins or toxoids) which cause a disease or condition to be prevented, treated or reversed. The homologously recombinant slow-growing bacteria can also be used to express enzymes, immunopotentiators, lymphokines, pharmacologic agents, antitumor agents (e.g., cytokines), or stress proteins (useful for evoking or enhancing an immune response or inducing tolerance in an autoimmune disease).
5 For example, homologously recombinant slow-growing mycobacteria of the present invention can express polypeptides or proteins which are growth inhibitors or are cytoidal for tumor cells (e.g., interferon α , β or γ , interleukins 1-7, tumor necrosis factor (TNF) α or β) and, thus, are
10 useful for treating certain human cancers (e.g., bladder cancers, melanomas). Homologously recombinant slow-growing mycobacteria of the present invention are also useful vehicles to elicit protective immunity in a host, such as a human or other vertebrate. They can be used to
15 produce humoral antibody immunity, cellular immunity and/or mucosal or secretory immunity. The antigens expressed by the homologously recombinant slow-growing mycobacteria, useful as vaccines or as diagnostic reagents, are also the subject of the present invention. In
20 addition, homologously recombinant slow-growing mycobacteria of the present invention are useful as vaccines in which the heterologous DNA introduced through homologous integration is not itself expressed, but acts to knock out a mycobacterial gene necessary for pathogenicity
25 of the slow-growing mycobacterium or its growth in vivo.
30

-10-

Such homologously recombinant slow-growing mycobacteria are useful as vaccines to provide protection against diseases caused by the corresponding wild-type mycobacterium or as a vaccine vehicle which contains a gene(s) 5 encoding an antigen(s) of a different pathogen(s) (e.g., as a vaccine to provide protection against an organism other than the corresponding wild-type mycobacterium or against a toxin or toxoid).

The vaccine of the present invention has important 10 advantages over presently available vaccines. For example, mycobacteria have adjuvant properties; they stimulate a recipient's immune system to respond to other antigens with great effectiveness. In addition, the mycobacterium stimulates long-term memory or immunity. This means that 15 a single (one time) inoculation can be used to produce long-term sensitization to protein antigens. Long-lasting T cell memory, which stimulates secondary antibody response neutralizing to the infectious agent or toxic. This is particularly useful, for example, against tetanus 20 and diphtheria toxins, pertussis, malaria, influenza, herpes viruses and snake venoms.

BCG in particular has important advantages as a vaccine vehicle. For example, it can be used repeatedly in an individual and has had a very low incidence of 25 adverse effects. In addition, BCG, as well as other mycobacteria, have a large genome (approximately 3×10^6 bp in length). As a result, a large amount of heterologous DNA can be accommodated within (incorporated into) the mycobacterial genome, which means that a large gene or 30 multiple genes (e.g., DNA encoding antigens for more than

-11-

one pathogen) can be inserted into genomic DNA, such as by homologous recombination.

In another embodiment of the present invention, the method of homologous recombination is used to manipulate the genome of a mycobacterium, such as *M. tuberculosis*, in order to attenuate the organism and render it more suitable for vaccine purposes (e.g., to render *M. tuberculosis* more suitable for use as a tuberculosis vaccine which would induce a protective response and minimize or reduce the risk of disease to the recipient). For example, a gene or genes encoding a product responsible for pathogenicity of the organism can be deleted or inactivated (e.g., by disruption). For *M. tuberculosis*, such genes include, but are not limited to, the *katG* gene, the *Hsp60* gene, *aroA*, *lysA*, *uraA* and the *M. tuberculosis* DNA associated with entry into and survival inside cells. The heterologous DNA used includes DNA homologous to genomic DNA of *M. tuberculosis*, which is introduced into genomic DNA in a location which results in deletion or disruption of the *M. tuberculosis* gene(s), resulting in attenuation of the *M. tuberculosis*.

Brief Description of the Figures

Figure 1 is a structural and functional map of the *M. bovis BCG uraA* locus, in which a restriction map of the *uraA* locus and the recombinant insert DNAs for several plasmids used to study this region are depicted. The relative positions of the BCG *uraA* gene and the portions of other genes identified are summarized graphically and

-12-

the ability of each recombinant to complement the E. coli pyrF mutant is indicated.

Figure 2 is the nucleic acid sequence of the BCG uraA locus (Seq ID No. 1) and the predicted protein products
5 (Seq ID No. 2).

Figure 3 is a schematic representation of integration by homologous recombination in BCG. The uraA locus in wild-type BCG (top), the transforming DNA (middle) and a BCG transformant in which the transforming DNA fragment
10 has integrated via homologous recombination (bottom) are represented.

Figure 4 is a schematic representation of the Southern analysis of the BCG transformant represented in Figure 3.

15 Figure 5 shows the results of Southern blot analysis of genomic DNA isolated from wild-type BCG (WT) and a BCG transformant (6015-9). The positions of DNA markers are indicated to the right and the apparent size of each of the hybridizing DNA bands is indicated to the left.

20 Detailed Description of the Invention

As described herein, Applicants have demonstrated introduction of heterologous DNA into slow-growing mycobacteria (transformation of heterologous DNA into slow-growing mycobacteria) and incorporation of heterologous
25 DNA at a homologous locus in genomic DNA of slow-growing mycobacterial (integration of heterologous DNA into the genomic DNA through homologous recombination). As a result, they have produced homologously recombinant slow-growing mycobacteria having heterologous DNA integrated at

-13-

a homologous locus in their genomic DNA. In particular, as described herein, Applicants have introduced heterologous DNA into M. bovis BCG (BCG) and demonstrated that it is present in the resulting homologously recombinant 5 BCG at a genomic location homologous with sequences present in the DNA construct transformed into the BCG. The DNA construct introduced into BCG by the method described herein included heterologous DNA containing the *uraA* locus (homologous DNA) and nonhomologous DNA (a selectable 10 marker gene); the heterologous homologous DNA flanked the nonhomologous DNA in the construct. Both the heterologous homologous DNA and the heterologous nonhomologous DNA in the DNA construct were shown to have integrated into the genome of the recipient mycobacterial cells at a homologous 15 locus (a genomic locus homologous with the DNA including and/or flanked by the homologous DNA in the DNA construct).

As a result of the work described herein, a method of transforming slow growing mycobacteria, a method of introducing heterologous DNA into genomic DNA of a slow growing mycobacterium through integration at a homologous locus, DNA constructs useful in the method of introducing heterologous DNA into a homologous locus in genomic DNA of a slow growing mycobacteria, homologously recombinant slow 25 growing mycobacteria which contain heterologous DNA at a homologous locus in their genomic DNA, a BCG gene encoding orotidine-5'-monophosphate decarboxylase (BCG OMP DCase) and homologously recombinant slow growing mycobacteria useful as vaccines are available. The following is a 30 description of the present method, DNA constructs and

vaccines, as well as the isolated BCG OMP DCase gene and its use.

The present invention includes an improved method of transforming slow growing mycobacteria. In the present 5 method, slow growing mycobacteria are subjected to electroporation in water, preferably after exposure to (culturing in the presence of) glycine prior to electroporation and preferably also while they are in mid-log growth. Slow growing mycobacteria to be transformed with 10 heterologous DNA are combined with the heterologous DNA (which can be plasmid/circular DNA or linear DNA) in water. The resulting combination is subjected to electroporation under conditions (e.g., potential, capacitance and time) sufficient for entry of the heterologous DNA 15 into the slow growing mycobacteria. Electroporation is carried out at approximately 2 to 2.5 kV potential and approximately 1 to 125 μ F capacitance for approximately 4 to 40 milliseconds. In a specific embodiment, slow growing mycobacterial cells are electroporated in water at 20 approximately 2.5 kV potential and approximately 25 μ F capacitance for 5-6 milliseconds. In a further embodiment, slow growing mycobacteria to be transformed are exposed to glycine (e.g., 1 to 2% glycine) by addition of glycine to culture medium prior to harvest of the cells. 25 In a particular embodiment, slow growing mycobacteria are exposed to 1.5% glycine, which is added to culture medium, for approximately 24 hours prior to harvest of the cells for transformation. In another embodiment, slow-growing mycobacteria are in mid-log growth when they are trans- 30 formed. The cells can also have been exposed to glycine,

-15-

as described above, prior to electroporation, although that is not necessary. The mid-log slow growing mycobacteria are combined with heterologous DNA to be introduced into them and subjected to electroporation in water, as 5 described above, resulting in transformation of the heterologous DNA into slow growing mycobacteria in the combination.

The heterologous DNA introduced into slow growing mycobacteria by the present method is DNA obtained from 10 any source other than the mycobacterium into which it is being introduced. It can be of viral, bacterial, myco-bacterial, invertebrate or vertebrate (including human and other mammalian) origin, can be obtained from other organisms, such as parasites, or can be produced to have the 15 same nucleic acid sequence as the DNA in its naturally occurring source. Alternatively, it can be modified DNA. The DNA introduced can be plasmid (circular) DNA or linear DNA. The heterologous DNA contains DNA homologous to a locus in genomic DNA of the recipient slow growing myco-bacteria, DNA nonhomologous to a locus in genomic DNA of 20 the recipient cells or both. It is possible to combine slow growing mycobacteria and a DNA construct in which the heterologous DNA is only nonhomologous DNA and carry out the present method of transformation, if the goal is to 25 transform slow growing mycobacteria with greater efficiency than is possible with existing methods. Heterologous DNA introduced in this manner will integrate randomly into genomic DNA.

In order to produce homologously recombinant slow 30 growing mycobacteria through homologous integration be-

-16-

tween mycobacterial genomic DNA and heterologous DNA, the DNA construct must include sufficient DNA homologous with mycobacterial DNA to cause integration of the construct into a homologous genomic locus. If only homologous DNA 5 is present in the DNA construct used (e.g., in a construct introduced in order to knock out or activate endogenous mycobacterial DNA), at least 400 bp of homologous DNA will generally be used. If the DNA construct includes homologous DNA (for directing or targeting introduction into 10 mycobacterial genomic DNA) and nonhomologous DNA (e.g., DNA encoding a product to be expressed in homologously recombinant slow growing mycobacteria), there is homologous DNA on both sides of (flanking both ends of) the nonhomologous DNA. In general, there will be at least 15 approximately 250 bp of homologous DNA on each side of the nonhomologous DNA, although shorter flanking homologous sequences can be used, provided that they are of sufficient length to undergo homologous recombination with genomic sequences, resulting in their introduction into 20 mycobacterial genomic DNA (alone or in conjunction with nonhomologous DNA with which the homologous DNA is present in the DNA construct). In the embodiment described in the examples, 1.5 kb of homologous DNA (1.5 kb of uraA flanking sequence) has been shown to result in homologous 25 integration, along with nonhomologous DNA, into the uraA locus of M. bovis BCG.

The homologous DNA present in the DNA construct can be any DNA homologous to DNA present in genomic DNA of the recipient slow growing mycobacterium. Specifically described herein is the isolation and sequencing of the M. 30

-17-

bovis BCG OMP DCASE gene (*uraA*) and its use to introduce heterologous nonhomologous DNA into *M. bovis* BCG genomic DNA at a homologous locus. As described in the examples, a BCG DNA fragment which included the OMP DCASE coding sequence was modified to remove the OMP DCASE coding sequence and replace it with heterologous nonhomologous DNA encoding a selectable marker gene (i.e., the Kan^r (aph) gene). Specifically, the DNA construct was made by removing the OMP DCASE coding sequence from a 4.4 kb BCG DNA fragment containing *uraA* and replacing it with the Kan^r gene (aph), to produce a DNA fragment in which the selectable marker gene is flanked by 1.5 kb *uraA* DNA (to direct homologous recombination or integration of the homologous DNA and, along with it, the nonhomologous DNA into mycobacterial genomic DNA). All or a portion of the OMP DCASE gene can be used, with similar modifications, as a component of a DNA construct including other heterologous nonhomologous DNA to be introduced into *M. bovis* BCG genomic DNA at the *uraA* locus. Alternatively, other *M. bovis* genes can be used as the heterologous homologous component of a DNA construct useful for introducing heterologous nonhomologous DNA into the mycobacterium. Similarly, DNA from other slow growing mycobacteria (e.g., *M. leprae*, *M. tuberculosis*, *M. avium*, *M. africanum*) can be incorporated into a DNA construct to be used for homologous recombination in the respective slow growing mycobacteria.

The heterologous nonhomologous DNA in the DNA construct introduced into slow growing mycobacteria by the present method can be any DNA which is expressed in the

-18-

slow-growing mycobacteria or which is not expressed in the recipient mycobacteria but alters mycobacterial protein expression or function. For example, the heterologous nonhomologous DNA can be DNA encoding an antigen(s) of a 5 pathogen or pathogens. A pathogen is any virus, micro-organism, other organism or substance (e.g., toxins, toxoids) which causes a disease or undesirable condition. Homologously recombinant slow growing mycobacteria which express a protein antigen(s) from malaria sporozoites, 10 malaria merozoites, diphtheria toxoid, tetanus toxoid, Leishmania, Salmonella, M. africanum, M. intracellulare, M. avium, treponema, pertussis, herpes virus, measles virus, mumps, Shigella, Neisseria, Borrelia, rabies, poliovirus, human immunodeficiency virus (HIV), Simian 15 immunodeficiency virus (SIV), snake venom, insect venom or vibrio cholera can be produced using the method of the present invention. Homologously recombinant M. bovis BCG, which, in a nonhomologously recombinant form, has long been successfully administered as a vaccine in humans can 20 be used. The DNA encoding the protein antigen(s) can be obtained from sources in which it naturally occurs or can be produced through known recombinant techniques or known chemical synthetic methods. For example, the DNA can be produced by genetic engineering methods, such as cloning 25 or by the polymerase chain reaction (PCR).

A multipurpose or multifunctional vaccine (one which contains and expresses heterologous DNA encoding antigens from more than one pathogen) can be produced by the present method. In this embodiment, one or more DNA constructs are used to introduce heterologous homologous DNA 30

-19-

and heterologous nonhomologous DNA (DNA encoding an antigen against which protection is desired) into the slow growing mycobacterium. If one construct is used, it includes DNA encoding the antigens of interest, flanked by 5 homologous DNA sufficient for introduction of the heterologous DNA into a homologous locus in the mycobacterium. More than one construct can be used; in this case, each includes homologous DNA and nonhomologous DNA encoding an antigen of interest. A multifunctional vaccine of the 10 present invention can be homologously recombinant BCG which contains, within its genomic DNA, a gene encoding an antigen for M. leprae, a gene encoding an antigen for M. tuberculosis, a gene encoding an antigen for malaria and a gene encoding an antigen for Leishmania; these sequences 15 are flanked by heterologous sequences homologous with BCG DNA and are introduced into the BCG genome by homologous integration.

It is not necessary that heterologous nonhomologous DNA be expressed by homologously recombinant slow growing 20 mycobacteria of the present invention or even that there be heterologous nonhomologous DNA present. For example, in one embodiment, heterologous nonhomologous DNA is incorporated into genomic DNA of slow growing mycobacteria for the purpose of inactivating an endogenous myco- 25 bacterial gene, such as a gene necessary for the pathogenicity of the mycobacterium. Any gene involved in metabolism necessary for pathogenicity of the slow growing mycobacterium (or for its growth in humans or other animals) but whose absence (e.g., from being knocked out) 30 does not prevent it from being cultured can be targeted

-20-

for inactivation. For example, the *aroA* gene of M. tuberculosis can be inactivated. In another embodiment, heterologous nonhomologous DNA is introduced in order to activate or turn on an endogenous mycobacterial gene. In 5 either case, the heterologous nonhomologous DNA need not be expressed.

Inactivation by disruption or deletion of a gene or genes associated with pathogenicity of M. tuberculosis can be carried out to produce attenuated M. tuberculosis which 10 is useful as a vaccine. There are a number of M. tuberculosis genes with interesting functions that can be knocked out (deleted) or disrupted to produce attenuated M. tuberculosis. These include *katG* (Heym, B. *et al.*, J. Bacteriol., 175:4255-4259 (1993); Zhang, Y. *et al.*, Nature, 358:591-593 (1992)), the "spare" mycobacterial *Hsp60* gene 15 (Hong, T.H. *et al.*, Proc. Natl. Acad. Sci., U.S.A., 90:2608-2612 (1993), *aroA* (Garbe, T. *et al.*, J. Bacteriol., 172:6774-6782 (1990), *lysA* (Andersen, A.B. *et al.*, Gene, 124:105-109 (1993), *uraA* (Aldovini, A. *et al.*, J. Bacteriol., 175:7282-7289 (1993) and the M. tuberculosis 20 DNA associated with entry and survival inside cells (Arruda, S. *et al.*, Science, 261:1454-1457 (1993)). This is not a complete list of candidate genes, but is provided as an example of the types of genes that can be targeted.

25 The *katG* gene is useful because 1) it is known that *katG* can be deleted without creating a lethal mutation and 2) deletion will permit selection for isoniazid resistance, allowing for a quick selection for recombinants that have undergone homologous recombination at the legitimate locus.

-21-

M. tuberculosis expresses two chaperonin-60 homologs (Hong, T.H. et al., Proc. Natl. Acad. Sci., U.S.A., 90:2608-2612 (1993), only one of which appears to be a major target of the immune response (the 65 kDa antigen), 5 and it is thought that the "65 kDa antigen" may contribute to pathogenesis. Knockout mutations in each of these two genes can be made to investigate whether they are lethal. Heterologous DNA is introduced into M. tuberculosis by the present method by combining the M. tuberculosis and heterologous DNA whose introduction into M. tuberculosis genomic 10 DNA associated with pathogenesis of the organism, thus resulting in attenuation of the pathogenicity of the recipient M. tuberculosis. The heterologous DNA can be DNA homologous to genomic M. tuberculosis DNA or DNA 15 homologous to genomic M. tuberculosis DNA and DNA which is not homologous to genomic M. tuberculosis.

Heterologous DNA can be homologous DNA only; it is not necessary that heterologous nonhomologous be present. For example, homologous DNA can be introduced into an 20 endogenous mycobacterial gene (such as one essential for the pathogenicity of a slow growing mycobacterium) in order to disrupt or inactivate that gene. This is particularly useful in those embodiments in which an attenuated or disabled mycobacterium is desired, such as for use as a 25 vaccine to elicit an immune response against the mycobacterium itself or as a vehicle to be used in a similar manner to that in which homologously recombinant BCG can be used (to express antigens of other pathogens).

Homologously recombinant slow growing mycobacteria of 30 the present invention can be administered by known methods

-22-

and a variety of routes (e.g., intradermally, intra-muscularly, intravenously). They are useful as vehicles in which the heterologous nonhomologous DNA is expressed and as modified slow grow mycobacteria (e.g., mycobacteria with reduced or abolished pathogenicity) which are disabled or attenuated and, thus, useful as vaccines.

The present invention will now be illustrated by the following examples, which are not to be considered limited in any way.

10 MATERIALS AND METHODS

Strains and plasmids. M. bovis BCG used for DNA isolation and subsequent construction of the recombinant BCG plasmid and λ gt11 libraries was the Montreal Strain, ATCC #35735. M. bovis BCG was grown in Middlebrook 7H9 media, supplemented with 0.05% Tween 80, as described in Aldovini and Young, Nature 351:479-482, (1991). E. coli strain Y1107 (pyrF::Mu trpam lacZam hsdR- m+ su-) was obtained from D. Botstein. Plasmids were propagated in the E. coli strain DH5 α from Bethesda Research Laboratories. E. coli cultures used for plasmid selection were grown in Luria Bertani broth or agar with 50 μ g/ml ampicillin. Phage M13 used for the production of single stranded DNA were propagated in E. coli strain JM101 from New England BioLabs. JM101 was grown in YT medium (Maniatis). Genomic libraries were generated using pUC19 from Bethesda Research Laboratories. Plasmid pY6002 (Husson et al.; J. Bacteriol., 172:519-524 1990) was the source of the 1.3 kb BamHI DNA fragment containing the amino-glycoside phosphotransferase gene aph.

-23-

Enzymes. Klenow fragment of E. coli DNA polymerase was supplied by Promega. T7 polymerase, and Taq polymerase (Sequenase and Taquence) were provided by United States Biochemical.

5 Recombinant DNA library construction. To isolate BCG DNA, cells were harvested by centrifugation, washed, and resuspended in 50 mM Tris (pH 8.0), 10 mM EDTA, 10% sucrose, and 0.5 mg/ml lysozyme, and incubated at 37 degrees for one hour. EDTA was then added to 1%, and the mixture 10 was incubated at room temperature for 15 minutes. Three phenol/chloroform extractions were performed, followed by RNase treatment, phenol/chloroform extraction, chloroform extraction and ethanol precipitation. The DNA was then resuspended in TE buffer, (10 mM Tris pH 7.5, 1mM EDTA).

15 To construct the plasmid library, the DNA was subjected to partial digestion with Sau3A and DNA fragments of 2-6 kb were isolated by agarose gel electrophoresis onto DE81 paper and eluted in buffer containing 10 mM Tris, HC1, 1M NaCl and 1 mM EDTA. The DNA fragments were 20 then phenol-chloroform extracted, ethanol precipitated and ligated into BamH1 digested, calf-intestinal phosphatase treated pUC19 plasmid vector. E. coli cells were transformed with the ligated mixture, and approximately 4×10^5 recombinants were obtained. Plasmid DNA was obtained from 25 the pool of transformed colonies using an alkaline lysis method.

The λ gt11 library was constructed using a procedure described by Young. (Young, R.A., et al., Proc. Natl. Acad. Sci., USA, 82:2583-2587 (1985)). Briefly, BCG 30 genomic DNA was subjected to random partial digestion with

- 24 -

DNase I, EcoRI linkers were added to the digestion products, and DNA fragments of 4-8 kb were isolated by agarose gel electrophoresis and electroelution. The DNA fragments were then ethanol precipitated and ligated into EcoRI-digested λ gt11 arms. The ligation mixture was packaged into λ heads and the packaging mixture was used to infect *E. coli*. Approximately 5×10^6 recombinants were obtained.

EXAMPLE 1.

10 Isolation of BCG OMP DCase gene by complementation and plasmid DNA manipulation. The BCG recombinant library was used to transform the *E. coli* strain Y1107. Twenty-one transformants capable of growing in the absence of uracil were isolated, of which six were chosen for further evaluation by restriction analysis. Plasmid DNA was isolated by alkaline lysis from cells grown in liquid culture, and restriction analysis indicated that all of these plasmids contained the same or very similar insert DNAs. One of these clones (pY6006) was used for further study (see 15 Figure 1). A 0.6 kb BamHI DNA fragment from pY6006 was used to screen the λ gt11 library, leading to the isolation of phage Y3030. This phage carries a 5.6 kb EcoRI BCG DNA insert containing the OMP DCase gene. This insert DNA was subcloned into pGEMz(f+) to generate pY6011. The 4.4 kb 20 SacI-EcoRI fragment of the Y3030 insert was subcloned into pUC19 to generate pY6014. Plasmid pY6015 was derived from pY6014 by replacing *uraA* sequences with the *aph* gene; a 1.15 kb HincII DNA fragment containing *uraA* sequences was removed by partial HincII digestion of pY6014 DNA, and it 25

-25-

was replaced with a 1.3 kb BamHI fragment containing aph from pY6002 that was blunt-ended with Klenow.

DNA Sequence analysis. The M. bovis BCG uraA gene was sequenced from the 4.4 kb SacI-EcoRI fragment of the 5 λ gt11 phage Y3030 cloned into M13 in both orientations. The same DNA fragment was subcloned into pUC19 to generate pY6014 for further manipulation. Single strand DNA for sequence analysis was prepared from M13 grown in JM101 (Viera and Messing, Methods Enzymol., 153:3-11 1987).

10 Both DNA strands were sequenced using the dideoxy-method of Sanger (Sanger et al., Proc. Natl. Acad. Sci. USA 74:5463-5467, 1977). Mycobacterial DNA has a high GC content, and two different strategies were used to reduce band compression and other artifacts due to high G+C 15 content. A subset of the reactions was carried out using Taq polymerase at high temperature (70°C). In addition, dGTP and dITP were used in independent sequence reactions (Kimsey and Kaiser, J. Biol. Chem. 267:819-824, 1992).

RESULTS

20 Isolation of the BCG OMP decarboxylase gene by genetic complementation. The complementation strategy employed to isolate the BCG OMP DCASE gene was similar to that employed previously to isolate the homologous gene in M. smegmatis (Husson et al., J. Bacteriol. 172:519-524, 25 1990). A recombinant library was constructed in the E. coli vector pUC19 using size selected BCG genomic DNA fragments from a partial SauIIIA digest. An E. coli pyrF mutant strain (Y1107) was transformed with this library and cells were plated on medium lacking uracil to select

- 26 -

for uracil prototrophs, and on rich medium containing ampicillin to ascertain the transformation frequency and to estimate the fraction of transformants that were able to complement the *E. coli* *pyrF* defect. Approximately 5 0.05% of the cells transformed with the recombinant library became uracil prototrophs. DNA clones were obtained from six colonies able to grow in the absence of uracil, and restriction analysis revealed that these clones contained the same insert DNA. One of these clones, pY6006, 10 was subjected to further study (Figure 1).

To identify, the portion of the 3.5 kb insert DNA pY6006 that was responsible for complementation, the 1.3-kb BamHI fragment of Tn903, which encodes aminoglycoside transferase (*aph*), was inserted into several 15 different sites in pY6006 insert DNA, the resultant plasmids were reintroduced into the *E. coli* *pyrF* mutant strain, and the ability of the new plasmids to complement the mutant phenotype was assessed as before (Figure 1). One of the three plasmids with insertion mutations, pY6006B, 20 lost the ability to complement the *pyrF* mutant phenotype, suggesting that sequences necessary for the complementing activity are located in the vicinity of the BamHI site that is disrupted in pY6006B.

Analysis of DNA sequences for the left end of pY6006 25 insert DNA (as diagrammed in Figure 1) revealed that the open reading frame of the pUC19 *lacZ* gene in this plasmid continues uninterrupted into an open reading frame for a polypeptide similar in sequence to OMP decarboxylase proteins. This preliminary data suggested that the left

-27-

end of pY6006 insert DNA encoded the amino-terminus of the BCG OMP decarboxylase protein.

For later experiments, it was important to have both the OMP decarboxylase gene and a substantial amount of 5 flanking sequences. To obtain genomic DNA that contains both the OMP decarboxylase gene and its flanking sequences, the 0.6 kb BamHI DNA fragment from pY6006 was used to probe a λ gt11 library, of *M. bovis* BCG DNA, as the λ gt11 library contains insert DNA fragments whose size, on 10 average, is larger (4-8 kb) than the plasmid library used to obtain pY6006. A lambda clone (Y3030) was isolated which contains a 5.6 kb EcoRI DNA insert that overlaps that of pY6006. The 5.6 kb EcoRI DNA fragment, and a 4.4 kb SacI-EcoRI subfragment, were subcloned into plasmid 15 vectors to generate pY6011 and pY6014, respectively (Figure 1). Both pY6011 and pY6014 were able to complement the defect of the *E. coli* *pyrF* mutant strain Y1107.

Sequence of the BCG OMP decarboxylase gene and flanking DNA. DNA fragments, from phage Y3030 insert DNA were 20 subcloned into M13 vectors and subjected to sequence analysis. Sequences were determined for both DNA strands, and most of the sequence reactions were duplicated with ITP replacing GTP to minimize artifacts due to the GC-rich nature of mycobacterial DNA. Figure 2 shows the sequences 25 obtained for the BCG OMP decarboxylase gene (uraA) (nucleotides 1691-2512) and for flanking DNA. The predicted BCG OMP decarboxylase protein sequence is 274 amino acids long, similar in size to other OMP decarboxylase proteins. The 274 amino acids of the predicted BCG OMP decarboxylase 30 protein are bracketed in Figure 2. When the BCG decarbox-

ylase protein sequence was used to screen the available databases for similar sequences, the results revealed that the BCG protein is closely related to the Myxococcus xanthus OMP DCases (Kirnsey and Kaiser, *J. Biol. Chem.*

5 267:819-824, 1992) and more distantly related to the other known prokaryotic and eukaryotic OMP DCases. Comparison of the BCG and M. xanthus OMP decarboxylases reveals that 40% of the amino acid residues are identical. In contrast, only 17% of the residues of the BCG and *E. coli* 10 proteins and 22% of the amino acids of the *M. xanthus* and *E. coli* proteins are identical, although there are a substantial number of conservative amino acid substitutions among these proteins. The relationship of *M. xanthus* OMP decarboxylase to homologues in other prokaryotes 15 and in eukaryotes was recently described in some detail (Kimsey and Kaiser, *J. Biol. Chem.* 267:819-824, 1992). This comparative sequence analysis revealed that there are four regions which are more highly conserved, and the predicted BCG OMP decarboxylase also shares this feature 20 with the other homologues. It is interesting to note that Mycobacteria and Myxococci both have GC-rich genomes, but this alone does not account for the degree of sequence conservation between the OMP decarboxylases from these two prokaryotes; rather, the two genera appear to be more 25 closely related to one another than either is to the other prokaryotes for which OMP decarboxylase sequence are available.

Further analysis of the BCG genomic DNA sequences revealed that the 1.7 kb sequence upstream of OMP decar- 30 boxylase coding sequences contains a single large open

reading frame. This open reading frame has no apparent beginning in the cloned DNA fragment, suggesting that it is the coding sequence for the carboxy-terminus of a larger protein. A screen of the sequence database revealed that the 497 amino acid residues of the predicted protein are highly homologous to the carboxyl termini of the large subunit of carbamoyl phosphate synthase. For example, the 497 amino acid carboxy terminus of the putative *M. bovis* BCG protein was 46% identical to the comparable segment of the *E. coli* carbamoyl phosphate synthase subunit, which is encoded by the *carB* gene (Nyunoya and Lusty, Proc. Natl. Acad. Sci. USA 80:4629-4633, 1983). Thus, the BCG *carB* gene appears to be located just upstream of *uraA*. This is interesting because both carbamoyl phosphate synthase and OMP decarboxylase are involved in pyrimidine biosynthesis. Carbamoyl phosphate synthase catalyzes the first reaction in pyrimidine biosynthesis, the production of carbamoyl phosphate, while OMP decarboxylase catalyzes the last step in the biosynthesis of UMP.

Analysis of BCG DNA sequences downstream of the *uraA* gene revealed a single large open reading frame that continues through the right end of the sequenced DNA fragment. This open reading frame predicts a protein of 501 amino acids. A search of the computer database revealed that the protein predicted by this ORF is similar to previously described proteins from *M. tuberculosis* and *M. leprae*. The predicted BCG protein is similar to a putative *M. tuberculosis* antigen encoded downstream of the gene for the 65 kDa antigen (Shinnick, T.M., J. Bacteriol. 169:1080-1088, 1987) and to a *M. leprae* antigen

-30-

that may be an integral membrane protein (Vega-Lopez et al., Infect. Immun. 61:2145-2154, 1993).

Southern analysis with whole genomic DNA revealed that there is a single copy of the *uraA* gene and flanking 5 DNA in the BCG genome (see below). The relative positions of the BCG *uraA* gene and the portions of other genes identified through sequence analysis are summarized graphically in Figure 1. The position of OMP decarboxylase sequences is consistent with the genetic analysis described above. The *aph* insertion mutations in plasmid 10 pY6006 that adversely affected complementation of the E. coli OMP decarboxylase mutant occurred within OMP decarboxylase coding sequences. Conversely, the *aph* insertion mutations that did not affect complementation of the E. coli OMP decarboxylase mutant occurred outside of the BCG 15 OMP decarboxylase coding sequences.

EXAMPLE 2

BCG transformation. BCG Pasteur (ATCC) was grown in log phase to an OD_{600} of 0.5 in Middlebrook medium. BCG cells 20 were harvested by centrifugation and washed twice with PBS (phosphate buffered saline) and resuspended in 1mM MgCl₂ (pH 7.2), 10% sucrose, 15% glycerol at a concentration of 10 OD_{600} per ml. 0.4 ml of BCG cells was mixed with 2 ug of plasmid DNA and electroporated in a 0.2 cm cuvette. 25 Electroporation settings were 2.5 kV potential and 25 μ F capacitance. After electroporation, cells were resuspended in 10 ml Middlebrook medium and incubated at 37C

-31-

for 2 hours before plating on Middlebrook agar containing 20 ug/ml kanamycin and, in some experiments, with uracil.

Southern blot analysis. Genomic DNAs from BCG strains were isolated as described above, digested with 5 restriction enzymes, subjected to agarose gel electrophoresis in the presence of ethidium bromide, transferred to nitrocellulose, and probed with DNA labelled with ^{32}P by random priming, all by standard procedure (Ausubel *et al.*, Current protocol in molecular biology (1987). Green 10 Publishing Associates and Wiley Interscience).

Introduction of foreign DNA into the BCG genome.

Previous attempts to obtain homologous recombination in *M. bovis* BCG have apparently not been successful (Kalpana *et al.*, Proc. Natl. Acad. Sci. USA 88:5433-5447, 1991; Young 15 and Cole, J. Bacteriol. 175:1-6, 1993). It is possible that the efficiency of transformation has an influence on the ability to obtain homologous recombination. To maximize the transformation efficiency of BCG, we investigated the effect of adding glycine to the culture medium prior 20 to harvesting cells for electroporation, as the presence of 1.5% glycine can affect the integrity of the cell wall and it seems to improve transformation efficiency in *M. smegmatis* (Mizuguchi and Takunaga, "Spheroplasts of Mycobacteria. 2. Infection of Phage and Its DNA on Glycine 25 Treated Mycobacteria and Spheroplasts", Med. Biol., 77:57 1968). In addition, we compared the efficiency of electroporation of BCG cells in water relative to buffer. The autonomously replicating plasmid pYUB12 (Snapper *et al.*, Mol. Microbiol. 4:1911-1919, 1988) was used to determine

-32-

how these variables affected the relative efficiencies of transformation. The results are summarized in the Table under Experiment 1. Transformation efficiencies were improved substantially by exposing cultures to 1.5% glycine for 24 hours prior to harvest, and by performing the 5 electroporation in water rather than in buffer.

-33-

TABLE. BCG Transformation Efficiencies

<u>Transforming DNA^a</u>	<u>Glycine Treatment^b</u>	<u>Electroporation Medium^c</u>	<u>Transformants/ug DNA</u>		
			<u>Expt 1</u>	<u>Expt 2</u>	<u>Expt 3</u>
pYUB12	-	Buffer	50	-	-
pYUB12	+	Buffer	250	-	-
pYUB12	-	Water	500	-	-
pYUB12	+	Water	10 ⁴	10 ⁴	10 ⁵
None	+	Water	8	6	35
p6015(I)	-	Buffer	-	4	-
p6015(I)	+	Buffer	-	22	-
p6015(I)	-	Water	-	39	-
p6015(I)	+	Water	-	98	500

^aThe intact autonomously replicating plasmid pYUB12 was used as a control and the linear insert DNA of plasmid pY6015 [pY6015(I)] was used as integrating DNA.

^bGlycine was added to 1.5% to BCG cultures 24 hours prior to transformation.

^cThe buffer is 1mM MgCl₂ (pH 7.2), 10% sucrose, 15% glycerol.

-34-

Experiments with linearized DNA molecules in yeast indicate that the ends of linear DNA molecules are recombinogenic; these ends may facilitate homologous integration by invading genomic DNA at homologous sites to initiate 5 recombination (Rothstein, R., Meth., Enzymol. 194:281-301 (1988)). The sequenced 4.4 kb BCG DNA fragment containing *UraA* was used to investigate whether cloned DNA sequences could integrate at the homologous locus in M. bovis BCG. To mark the DNA fragment, the OMP decarboxylase coding 10 sequence was replaced with a kanamycin-resistance gene (aph) to create pY6015 (Figure 3). This left intact approximately 1.5 kb of *UraA* flanking sequences that could be used to direct homologous integration. The transformation experiment described above for plasmid pYUB12 was 15 repeated with pY6015 insert DNA, and the results are summarized in the Table under Experiment 2. Again, transformation efficiencies were improved substantially by exposing cultures to 1.5% glycine for 24 hours prior to harvest, and by performing the electroporation in water 20 rather than in buffer. However, because the transformation efficiencies obtained with the linear DNA were low, we made one additional attempt to improve these efficiencies.

Cultures of *M. bovis* BCG and other slow growing 25 mycobacteria contain large numbers of cells that are inviable or that have an exceedingly long lag time after plating. Some investigators have suggested that mycobacterial cells have an unusual ability to enter and maintain a dormant state, even when nutrients are available (Young 30 and Cole, "Leprosy, Tuberculosis, and the New Genetics",

-35-

J. Bacteriol., 175:1-6 1993). We reasoned that maintenance of BCG cultures in mid-log growth might maximize the fraction of cells that were undergoing DNA synthesis and were competent to take up DNA and to incorporate it into homologous sites in the genome. A third experiment was performed, in which BCG cultures were diluted approximately 1:4 every two days over a two-month period to ensure persistent log-phase growth before transformation. The results in the Table indicate that this approach produces a significant increase in the number of transformants obtained with either the autonomously replicating vector or the linear DNA fragment.

Ten of the BCG colonies obtained in the third experiment were selected for further study after growing to adequate size for picking (24 days after plating). The ten transformants were colony purified, and DNA was prepared from each. DNA preparations from the wild type strain and the ten transformants were digested with a variety of restriction endonucleases and Southern analysis revealed that the kanamycin-resistant BCG transformants all contained vector DNA integrated into the genome. In two of the ten transformants, the transforming DNA had integrated at the homologous locus. Figure 5 shows representative results from Southern analysis of the wild type strain and one of the BCG recombinants in which the cloned DNA integrated at the homologous locus.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using not more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

- 36 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Whitehead Institute for Biomedical Research
(B) STREET: Nine Cambridge Center
(C) CITY: Cambridge
(D) STATE/PROVINCE: Massachusetts
(E) COUNTRY: USA
(F) POSTAL CODE/ZIP: 02142
(G) TELEPHONE:
(I) TELEFAX:

(ii) TITLE OF INVENTION: Homologously Recombinant Slow Growing Mycobacteria and Uses Therefor

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Hamilton, Brook, Smith & Reynolds, P.C.
(B) STREET: Two Militia Drive
(C) CITY: Lexington
(D) STATE: MA
(E) COUNTRY: USA
(F) ZIP: 02173

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 08/095,734
(B) FILING DATE: 22-JUL-1993

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Granahan, Patricia
(B) REGISTRATION NUMBER: 32,227
(C) REFERENCE/DOCKET NUMBER: WHI93-11MA PCT

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 617-861-6240
(B) TELEFAX: 617-861-9540

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4394 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

- 37 -

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAGCTCGACC	CCGCCGCCGA	AACAGAGGTG	GCCCCGCAGA	CCGAAAGGCC	CAAGGTGCTG	60
ATCCTCGGTT	CGGGGCCCAA	TCGGATCGGC	CAGGGTATCG	AGTTCGACTA	CAGCTGCGTA	120
CACGCGGCAA	CCACGTTGAG	CCAGGCTGGC	TTTGAGACCG	TGATGGTCAA	CTGCAACCCG	180
GAGACCATGG	TGTCCACCGA	CTTCGACACC	GC GGACAGGT	TGTACTTCGA	GCCGTTGACG	240
TTCGAGGACG	TCTTGGAGGT	CTACCACGCC	GAAATGGAAT	CCGGTAGCGG	TGGCCCGGGA	300
GTGGCCGGCG	TCATCGTGCA	GCTCGGCGGC	CAGACCCCGC	TCGGCTGGCG	CACCGGCTCG	360
CCGACGCCGG	GTCCCGCTCG	TGGCACCCA	CCGGAGGCCA	TCGACCTGGC	CGAGGATGCG	420
GCCGTTCGGC	GACCTGCTGA	GCGAGGACTG	CCGGCGCCAA	AGTACGGCAC	CGCAACCACT	480
TTCGCCCAGG	CCCGCCGGAT	CGCCGAGGAG	ATCGGCTATC	CGGTGCTGGT	GCGGCCGTG	540
TATGTGCTCG	GTGGTCGCGG	CATGGAGATC	GTGTATGACG	AAGAAACGTT	GCAGGGCTAC	600
ATCACCCGCG	CCACTCAGCT	ATCCCCGAA	CACCCGGTGC	TCGTGCACCG	CTTCCTCGAG	660
GACGCGGTG	AGATCGACGT	CGACGCTCTG	TGTGATGGCG	CCGAGGTCTA	TATCGGCGGA	720
ATCATGGAGC	ACATCGAGGA	GGCCGGCATC	CACTCCGGTG	ACTCGGCCTG	TGCGCTGCCA	780
CCGGTCACGT	TGGGCCGCAG	CGACATCGAG	AAGGTGCGTA	AGGCCACTGA	AGCCATTGCG	840
CATGGCCTCG	GC GTGGTGGG	GCTGCTAAC	GTGCAGTCCG	CGCTCAAGGA	TGACGTGCTC	900
TACGTCCCTGG	AAGCCAACCC	GAGAGCGAGC	CGTACCGTTC	CGTTTGTATC	CAAGGCCACA	960
GCGGTGCCAC	TCGCCAAGGC	ATGCGCCCGG	ATCATGTTGG	GCGCCACCAT	TGCCAGCTG	1020
CGCGCCGAAG	GCTTGCTGGC	GGTCACCGGG	GATGGCGCCC	ACGCGCGCG	AAACGCCCGC	1080
ATCGCGGTCA	ACCAGGCCGT	GTTGCCGTTT	CACCGGTTCC	GGCGCGCCGA	CGGGGCCGCG	1140
ATCGACTCGC	TACTCGGCC	GGAGATGAAA	TCGACCGGCG	AGGTGATGGG	CATCGACCGC	1200
GA CTT CGGCA	GCCGGTTCGC	CAAGAGCCAG	ACCGCCGCCT	ACGGGTGCGT	GCCGGCCCG	1260
GGCACAGTGT	TCGTGTCGGT	GGCCAACCGG	GACAAGCGGT	CGCTGGTGT	TCCGGTCAA	1320
CCGATTGGCC	CACCTGGGTT	TTCGCGTCCT	TGCCACCGAA	GCACCGCAGA	GATCTTGC	1380
CGCAACGGTA	TTCCCTGCGA	CGACGTCCGC	AAACATTTCG	AGCCGGCGCA	GCCCGGCCGC	1440
CCCACAAATGT	CGGCGGTGGA	CGCGATCCGA	GCCGGCGAGG	TCAACATGGT	GATCAACACT	1500
CCCTATGGCA	ACTCCGGTCC	GCGCATCGAC	GGCTATGAGA	TCCGTTCGGC	GGCGGTGGCC	1560
GGCAACATCC	CGTGCATCAC	CACGGTGCAG	GGCGCATCCG	CCGCCGTGCA	GGGGATAGAG	1620

GCCGGGATCC	CGGGCGACAT	CGGGGTGCGC	TCCCTGCAGG	AGCTGCACCG	GGTGATCGGG	1680
GGCGTCGAGC	GGTGACCGGG	TTCGGTCTCC	GGTTGGCCGA	GGCAAAGGCA	CGCCGCGGCC	1740
CGTTGTGTCT	GGGCATCGAT	CCGCATCCCC	AGCTGCTGCG	GGGCTGGGAT	CTGGCGACCA	1800
CGGCCGACGG	GCTGGCCGCG	TTCTGCGACA	TCTGCGTACG	GGCCTTCGCT	GATTCGCGG	1860
TGGTCAAACC	GCAGGTGGCG	TTTTTGAGT	CATACGGGC	TGCCGGATTC	GCGGTGCTGG	1920
AGCGCACCAT	CGCGGAACCTG	CGGGCCGCG	ACGTGCTGGT	GTTGGCCGAC	GCCAAGCGCG	1980
GCGACATTGG	GGCGACCATG	TCGGCGTATG	CGACGGCCTG	GGTGGGCGAC	TCGCCGCTGG	2040
CCGCCGACGC	CGTGACGGCC	TCGCCCTATT	TGGGCTTCGG	TTCGCTGCGG	CCGCTGCTAG	2100
AGGTGCGGGC	CGCCCACGGC	CGAGGGGTGT	TCGTGCTGGC	GGCCACCTCC	AATCCCGAGG	2160
GTGCGGCGGT	GCAGAATGCC	GCCGCCGACG	GCCGCAGCGT	GGCCCAGTTG	GTCGTGGACC	2220
AGGTGGGGGC	GGCCAACGAG	GGCGCAGGAC	CCGGGCCCCG	ATCCATCGGC	GTGGTGTGCG	2280
GCGAACCGGC	GCCACAGGCC	CCCGATCTCA	GCGCCTTCAC	CGGGCCGGTG	CTGGTGCCCG	2340
GCGTGGGGGT	GCAGGGCGGG	CGCCCGGAGG	CGCTGGGCGG	TCTGGGCGGG	GCCGCATCGA	2400
GCCAGCTGTT	GCCCCGCGGTG	GCGCGCGAGG	TCTTGCGGGC	CGGGCCCCGC	GTGCCCGAAT	2460
TGCGCGCCGC	GGCGAACCGG	ATGCGCGATG	CCGTCGCCTA	TCTCGCTGCC	GTGTAGCGGG	2520
TGCCCTGCCA	CCGCGCCGCT	AAATCCCACC	AGCATGGGT	GGTGAGGCCA	GCGCTCGTGT	2580
GACCAAACTC	ACCGCCCTGG	GCCGTCGTCA	CGCTGTGTTA	ACCTCTCGTT	CAAATGATAT	2640
TCATATTCAA	TAGTGGCGCT	AAAGTGTCCGG	TTGAATCCCC	GTTGAACCCC	CAACAGATGG	2700
AGTCTGTGTC	GTGACGTTGC	GAGTCGTTCC	CGAAAGCCTG	GCAGGCGCCA	GCGCTGCCAT	2760
CGAACAGCTG	ACCGCTCGCC	TGGCCGCCGC	GCACGCCGCG	GGGGCCCCGT	TTATCGCGGC	2820
GGTCATCCCG	CCTGGGTCGG	ACTCGGTTTC	GGTGTGCAAC	GCCGTTGAGT	TCAGCGTTCA	2880
CGGTAGTCAG	CATGTGGCAA	TGGCCGCTCA	GGGGGTTGAG	GAGCTCGGCC	GCTCGGGGGT	2940
CGGGGTGGCC	GAATCGGGTG	CCAGTTATGC	CGCTAGGATG	CGCTGGCGGC	GGCGTCGTAT	3000
CTCAGCGGTG	GGCTATGACC	GAGCCGTGGA	TAGCCTTCCC	TCCCGAGGTG	CACTCGGCCA	3060
TGCTGAACTA	CGGTGCGGGC	GTTGGGCCGA	TGTTGATCTC	CGCCACGCAG	AATGGGGAGC	3120
TCAGCGCCCA	ATACGCAGAA	GCGGCATCCG	AGGTCGAGGA	ATTGTTGGGG	GTGGTGGCCT	3180
CCGAGGGATG	GCAGGGGCAA	GCCGCCGAGG	CGTTAGTCGC	CGCGTACATG	CCGTTTCTGG	3240
CGTGGCTGAT	CCAAGCCAGC	GCCGACTGCC	TGGAAATGGC	CGCCCAGCAA	CACGCCGTCA	3300
TCGAGGCCTA	CACTGCCGCG	GTAGAGCTGA	TGCCTACTCA	GGTCGAACCTG	GCCGCCAAC	3360
AAATCAAGCT	CGCGGTGTTG	GTAGCGACCA	ATTTCTTGG	CATCAACACC	ATTCCCATTG	3420

- 39 -

CGATCAATGA GGCGGAGTAC GTGGAGATGT GGGTTGGGC CGCCACCACG ATGGCGACCT	3480
ATTCAACAGT CTCCAGATCG GCGCTCTCCG CGATGCCGCA CACCAGCCCC CCGCCGCTGA	3540
TCCTGAAATC CGATGAAC TG CTCCCCGACA CGGGGGAGGA CTCCGATGAA GACGCCACA	3600
ACCATGGCGG TCACAGTCAT GGC GGTCACG CCAGGATGAT CGATAACTTC TTTGCCGAAA	3660
TCCTGCGTGG CGTCAGCGCG GGCGCATTG TTTGGGACCC CGTCAACGGC ACCCTCAACG	3720
GACTCGACTA CGACGATTAC GTCTACCCCG GTCACGCGAT CTGGTGGCTG GCTCGAGGCC	3780
TCGAGTTTT TCAGGATGGT GAACAATTG GCGAACTGTT GTTCACCAAT CCGACTGGGG	3840
CTTTTCAGTT CCTCCTCTAC GTCGTTGTGG TGGATTGCC GACGCACATA GCCCAGATCG	3900
CTACCTGGCT GGGCCAGTAC CCGCAGTTGC TGTCGGCTGC CCTCACTGGC GTCATCGCCC	3960
ACCTGGGAGC AATAACTGGT TTGGCGGGCC TATCCGGCCT GAGGCCATT CCGTCTGCTG	4020
CGATACCCGC CGTTGTACCG GAGCTGACAC CCGTCGCGGC CGCGCCGCCT ATGTTGGCGG	4080
TCGCCGGGGT GGGCCCTGCA GTCGCCGCGC CGGGCATGCT CCCCACCTCA GCACCCGCAC	4140
CGGCGGCAGC GGCGGGCGCC ACCGCAGCCG GCCCGACGCC GCCGGCGACT GGTTTCGGAG	4200
GGCTTCCCAGC CCTACCTGGT CGGCGGTGGC GGCCCAGGAA TAGGGTTCGG CTCGGGACAG	4260
TCGGCCCCACG CCAAGGCCGC GGCGTCCGAT TCCGCTGCAG CCGAGTCGGC GGCCCAGGCC	4320
TCGGCGCGTG CGCAGGCCGC TGCTGCACGG CGGGGCCGCT CGGCGGCAAG GCACGTGGCC	4380
ATCGTGACGA ATTC	4394

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1271 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Glu	Leu	Asp	Pro	Ala	Ala	Glu	Thr	Glu	Val	Ala	Pro	Gln	Thr	Glu	Arg
1				5					10				15		
Pro	Lys	Val	Leu	Ile	Leu	Gly	Ser	Gly	Pro	Asn	Arg	Ile	Gly	Gln	Gly
				20				25				30			
Ile	Glu	Phe	Asp	Tyr	Ser	Cys	Val	His	Ala	Ala	Thr	Thr	Leu	Ser	Gln
	35					40					45				
Ala	Gly	Phe	Glu	Thr	Val	Met	Val	Asn	Cys	Asn	Pro	Glu	Thr	Met	Val
	50					55					60				

- 40 -

Ser Thr Asp Phe Asp Thr Ala Asp Arg Leu Tyr Phe Glu Pro Leu Thr
65 70 75 80

Phe Glu Asp Val Leu Glu Val Tyr His Ala Glu Met Glu Ser Gly Ser
85 90 95

Gly Gly Pro Gly Val Ala Gly Val Ile Val Gln Leu Gly Gly Gln Thr
100 105 110

Pro Leu Gly Trp Arg Thr Gly Ser Pro Thr Pro Gly Pro Ala Arg Gly
115 120 125

His Pro Pro Glu Ala Ile Asp Leu Ala Glu Asp Ala Ala Val Arg Arg
130 135 140

Pro Ala Glu Arg Gly Leu Pro Ala Pro Lys Tyr Gly Thr Ala Thr Thr
145 150 155 160

Phe Ala Gln Ala Arg Arg Ile Ala Glu Glu Ile Gly Tyr Pro Val Leu
165 170 175

Val Arg Pro Ser Tyr Val Leu Gly Gly Arg Gly Met Glu Ile Val Tyr
180 185 190

Asp Glu Glu Thr Leu Gln Gly Tyr Ile Thr Arg Ala Thr Gln Leu Ser
195 200 205

Pro Glu His Pro Val Leu Val His Arg Phe Leu Glu Asp Ala Val Glu
210 215 220

Ile Asp Val Asp Ala Leu Cys Asp Gly Ala Glu Val Tyr Ile Gly Gly
225 230 235 240

Ile Met Glu His Ile Glu Glu Ala Gly Ile His Ser Gly Asp Ser Ala
245 250 255

Cys Ala Leu Pro Pro Val Thr Leu Gly Arg Ser Asp Ile Glu Lys Val
260 265 270

Arg Lys Ala Thr Glu Ala Ile Ala His Gly Ile Gly Val Val Gly Leu
275 280 285

Leu Asn Val Gln Ser Ala Leu Lys Asp Asp Val Leu Tyr Val Leu Glu
290 295 300

Ala Asn Pro Arg Ala Ser Arg Thr Val Pro Phe Val Ser Lys Ala Thr
305 310 315 320

Ala Val Pro Leu Ala Lys Ala Cys Ala Arg Ile Met Leu Gly Ala Thr
325 330 335

Ile Ala Gln Leu Arg Ala Glu Gly Leu Leu Ala Val Thr Gly Asp Gly
340 345 350

Ala His Ala Ala Arg Asn Ala Pro Ile Ala Val Asn Gln Ala Val Leu
355 360 365

Pro Phe His Arg Phe Arg Arg Ala Asp Gly Ala Ala Ile Asp Ser Leu
370 375 380

-41-

Leu Gly Pro Glu Met Lys Ser Thr Gly Glu Val Met Gly Ile Asp Arg
385 390 395 400

Asp Phe Gly Ser Arg Phe Ala Lys Ser Gln Thr Ala Ala Tyr Gly Ser
405 410 415

Leu Pro Ala Gln Gly Thr Val Phe Val Ser Val Ala Asn Arg Asp Lys
420 425 430

Arg Ser Leu Val Phe Pro Val Lys Arg Leu Ala His Leu Gly Phe Arg
435 440 445

Val Leu Ala Thr Glu Ala Pro Gln Arg Ser Cys Ala Ala Thr Val Phe
450 455 460

Pro Ala Thr Thr Ser Ala Asn Ile Ser Ser Arg Arg Ser Pro Ala Ala
465 470 475 480

Pro Gln Cys Arg Arg Trp Thr Arg Ser Glu Pro Ala Arg Ser Thr Trp
485 490 495

Met Thr Gly Phe Gly Leu Arg Leu Ala Glu Ala Lys Ala Arg Arg Gly
500 505 510

Pro Leu Cys Leu Gly Ile Asp Pro His Pro Glu Leu Leu Arg Gly Trp
515 520 525

Asp Leu Ala Thr Thr Ala Asp Gly Leu Ala Ala Phe Cys Asp Ile Cys
530 535 540

Val Arg Ala Phe Ala Asp Phe Ala Val Val Lys Pro Gln Val Ala Phe
545 550 555 560

Phe Glu Ser Tyr Gly Ala Ala Gly Phe Ala Val Leu Glu Arg Thr Ile
565 570 575

Ala Glu Leu Arg Ala Ala Asp Val Leu Val Leu Ala Asp Ala Lys Arg
580 585 590

Gly Asp Ile Gly Ala Thr Met Ser Ala Tyr Ala Thr Ala Trp Val Gly
595 600 605

Asp Ser Pro Leu Ala Ala Asp Ala Val Thr Ala Ser Pro Tyr Leu Gly
610 615 620

Phe Gly Ser Leu Arg Pro Leu Leu Glu Val Ala Ala Ala His Gly Arg
625 630 635 640

Gly Val Phe Val Leu Ala Ala Thr Ser Asn Pro Glu Gly Ala Ala Val
645 650 655

Gln Asn Ala Ala Ala Asp Gly Arg Ser Val Ala Gln Leu Val Val Asp
660 665 670

Gln Val Gly Ala Ala Asn Glu Ala Ala Gly Pro Gly Pro Gly Ser Ile
675 680 685

- 42 -

Gly Val Val Val Gly Ala Thr Ala Pro Gln Ala Pro Asp Leu Ser Ala
690 695 700

Phe Thr Gly Pro Val Leu Val Pro Gly Val Gly Val Gln Gly Gly Arg
705 710 715 720

Pro Glu Ala Leu Gly Gly Leu Gly Gly Ala Ala Ser Ser Gln Leu Leu
725 730 735

Pro Ala Val Ala Arg Glu Val Leu Arg Ala Gly Pro Gly Val Pro Glu
740 745 750

Leu Arg Ala Ala Gly Glu Arg Met Arg Asp Ala Val Ala Tyr Leu Ala
755 760 765

Ala Val Met Trp Gln Trp Pro Leu Arg Gly Leu Arg Ser Ser Ala Ala
770 775 780

Arg Gly Ser Gly Trp Pro Asn Arg Val Pro Val Met Pro Leu Gly Cys
785 790 795 800

Ala Gly Gly Gly Val Val Ser Gln Arg Trp Ala Met Thr Glu Pro Trp
805 810 815

Ile Ala Phe Pro Pro Glu Val His Ser Ala Met Leu Asn Tyr Gly Ala
820 825 830

Gly Val Gly Pro Met Leu Ile Ser Ala Thr Gln Asn Gly Glu Leu Ser
835 840 845

Ala Gln Tyr Ala Glu Ala Ala Ser Glu Val Glu Glu Leu Leu Gly Val
850 855 860

Val Ala Ser Glu Gly Trp Gln Gly Gln Ala Ala Glu Ala Leu Val Ala
865 870 875 880

Ala Tyr Met Pro Phe Leu Ala Trp Leu Ile Gln Ala Ser Ala Asp Cys
885 890 895

Val Glu Met Ala Ala Gln Gln His Ala Val Ile Glu Ala Tyr Thr Ala
900 905 910

Ala Val Glu Leu Met Pro Thr Gln Val Glu Leu Ala Ala Asn Gln Ile
915 920 925

Lys Leu Ala Val Leu Val Ala Thr Asn Phe Phe Gly Ile Asn Thr Ile
930 935 940

Pro Ile Ala Ile Asn Glu Ala Glu Tyr Val Glu Met Trp Val Arg Ala
945 950 955 960

Ala Thr Thr Met Ala Thr Tyr Ser Thr Val Ser Arg Ser Ala Leu Ser
965 970 975

Ala Met Pro His Thr Ser Pro Pro Leu Ile Leu Lys Ser Asp Glu
980 985 990

Leu Leu Pro Asp Thr Gly Glu Asp Ser Asp Glu Asp Gly His Asn His
995 1000 1005

-43-

Gly Gly His Ser His Gly Gly His Ala Arg Met Ile Asp Asn Phe Phe
1010 1015 1020

Ala Glu Ile Leu Arg Gly Val Ser Ala Gly Arg Ile Val Trp Asp Pro
1025 1030 1035 1040

Val Asn Gly Thr Leu Asn Gly Leu Asp Tyr Asp Asp Tyr Val Tyr Pro
1045 1050 1055

Gly His Ala Ile Trp Trp Leu Ala Arg Gly Leu Glu Phe Phe Gln Asp
1060 1065 1070

Gly Glu Gln Phe Gly Glu Leu Leu Phe Thr Asn Pro Thr Gly Ala Phe
1075 1080 1085

Gln Phe Leu Leu Tyr Val Val Val Asp Leu Pro Thr His Ile Ala
1090 1095 1100

Gln Ile Ala Thr Trp Leu Gly Gln Tyr Pro Gln Leu Leu Ser Ala Ala
1105 1110 1115 1120

Leu Thr Gly Val Ile Ala His Leu Gly Ala Ile Thr Gly Leu Ala Gly
1125 1130 1135

Leu Ser Gly Leu Ser Ala Ile Pro Ser Ala Ala Ile Pro Ala Val Val
1140 1145 1150

Pro Glu Leu Thr Pro Val Ala Ala Ala Pro Pro Met Leu Ala Val Ala
1155 1160 1165

Gly Val Gly Pro Ala Val Ala Ala Pro Gly Met Leu Pro Ala Ser Ala
1170 1175 1180

Pro Ala Pro Ala Ala Ala Gly Ala Thr Ala Ala Gly Pro Thr Pro
1185 1190 1195 1200

Pro Ala Thr Gly Phe Gly Leu Pro Ala Leu Pro Gly Arg Arg Trp
1205 1210 1215

Arg Pro Arg Asn Arg Val Arg Leu Gly Thr Val Gly Pro Arg Gln Gly
1220 1225 1230

Arg Gly Val Arg Phe Arg Cys Ser Arg Val Gly Gly Pro Gly Leu Gly
1235 1240 1245

Ala Cys Ala Gly Ala Cys Cys Thr Ala Gly Pro Leu Gly Gly Lys Ala
1250 1255 1260

Arg Gly His Arg Asp Glu Phe
1265 1270

- 44 -

CLAIMS

1. A method of transforming a slow-growing mycobacterium with heterologous DNA, comprising the steps of:
 - 5 a) combining a slow-growing mycobacterium and heterologous DNA to be transformed into the slow-growing mycobacterium, thereby producing a combination; and
 - b) subjecting the combination produced in (a) to electroporation in water, under conditions sufficient for introduction of the heterologous DNA 10 into slow-growing mycobacterium, thereby producing a slow-growing mycobacterium transformed with the heterologous DNA.
- 15 2. The method of Claim 1 wherein the slow-growing mycobacterium of (a) have been exposed to glycine, prior to being combined with the heterologous DNA.
- 20 3. The method of Claim 2 wherein the slow-growing mycobacterium are exposed to approximately 1.5% glycine present in culture medium in which the slow-growing mycobacterium is growing.
4. The method of Claim 1 in which the slow-growing mycobacterium is continuously propagated in mid-log phase.

-45-

5. The method of Claim 1 wherein the slow-growing mycobacterium is selected from the group consisting of:
Mycobacterium bovis BCG, Mycobacterium tuberculosis,
Mycobacterium leprae, Mycobacterium avium, Mycobacterium africanum and Mycobacterium intracellulare.
6. The method of Claim 5 wherein the heterologous DNA comprises DNA homologous to genomic DNA of the slow-growing mycobacterium combined in step (a) with the heterologous DNA.
- 10 7. The method of Claim 6 wherein the heterologous DNA is introduced into the uraA locus of the slow-growing mycobacterium.
8. The method of Claim 6 wherein the heterologous DNA additionally comprises DNA which is not homologous to
15 genomic DNA of the slow-growing mycobacterium combined in step (a) with the heterologous DNA.
9. The method of Claim 8 wherein the heterologous DNA is introduced into the uraA locus of the slow-growing mycobacterium.
- 20 10. The method of Claim 6 wherein the slow-growing mycobacterium is Mycobacterium tuberculosis and the DNA homologous to genomic DNA of the slow-growing mycobacterium is introduced into genomic DNA in a location which results in attenuation of the Mycobacterium tuberculosis.

-46-

11. The method of Claim 6 wherein the slow-growing myco-
bacterium is M. tuberculosis and the heterologous DNA
comprises DNA homologous to genomic DNA of M. tuber-
closis selected from the group consisting of: the

5 katG gene, the Hsp60 gene, aroA, lysA, uraA and M.
tuberculosis DNA associated with entry into and
survival inside cells.

12. A method of producing a heterologously recombinant
slow-growing mycobacterium having heterologous DNA
10 incorporated into genomic DNA at a homologous locus,
comprising the steps of:

15 a) combining a slow-growing mycobacterium and he-
terologous DNA to be transformed into the slow-
growing mycobacterium, the heterologous DNA
comprising DNA homologous to genomic DNA of the
slow-growing mycobacterium, thereby producing a
combination; and

20 b) subjecting the combination produced in (a) to
electroporation in water, under conditions suf-
ficient for introduction of the heterologous DNA
into the slow-growing mycobacterium and inte-
gration into genomic DNA of the slow-growing
mycobacterium at a homologous locus,
thereby producing a homologously recombinant slow-
25 growing mycobacterium having heterologous DNA incor-
porated into its genomic DNA at a homologous locus.

-47-

13. The method of Claim 12 wherein the slow-growing mycobacterium of (a) has been exposed to glycine, prior to being combined with the heterologous DNA.
14. The method of Claim 13 wherein the slow-growing mycobacterium is exposed to approximately 1.5% glycine present in culture medium in which the slow-growing mycobacterium is growing.
15. The method of Claim 12 in which the slow-growing mycobacterium is continuously propagated in mid-log phase.
16. The method of Claim 12 wherein the slow-growing mycobacterium is selected from the group consisting of: Mycobacterium bovis BCG, Mycobacterium tuberculosis, Mycobacterium leprae, Mycobacterium avium, Mycobacterium africanum and Mycobacterium intracellulare.
17. The method of Claim 12 wherein the heterologous DNA additionally comprises DNA which is not homologous to genomic DNA of the slow-growing mycobacterium combined in step (a) with the heterologous DNA.
18. The method of Claim 17 wherein the slow-growing mycobacterium is Mycobacterium bovis BCG and the DNA homologous to genomic DNA of the slow-growing mycobacterium is DNA contained in the Mycobacterium bovis BCG orotidine-5- monophosphate decarboxylase gene locus or its flanking sequences.

19. A method of producing attenuated Mycobacterium tuberculosis, comprising inactivating or deleting in Mycobacterium tuberculosis DNA selected from the group consisting of: the katG gene, the Hsp60 gene, aroA, lysA, uraA and DNA associated with entry into and survival inside cells of Mycobacterium tuberculosis.
- 5
20. A method of producing attenuated Mycobacterium tuberculosis comprising introducing into M. tuberculosis heterologous DNA by:
 - a) combining Mycobacterium tuberculosis and heterologous DNA, thereby producing a combination; and
 - b) subjecting the combination produced in a) to electroporation in water, under conditions sufficient for introduction of the heterologous DNA into the Mycobacterium tuberculosis,
- 10
- 15
- wherein the heterologous DNA is homologous to Mycobacterium tuberculosis genomic DNA selected from the group consisting of: the katG gene, the Hsp60 gene, aroA, lysA, uraA and M. tuberculosis DNA associated with entry into and survival inside cells and is introduced into genomic DNA of the Mycobacterium tuberculosis, thereby deleting or disrupting the
- 20
- 25
- corresponding Mycobacterium tuberculosis genomic DNA.
21. The method of Claim 20 wherein the Mycobacterium tuberculosis has been exposed to glycine prior to being combined with the heterologous DNA.

- 49 -

22. The method of Claim 21 wherein the Mycobacterium tuberculosis are exposed to approximately 1.5% glycine present in culture medium in which the Mycobacterium tuberculosis is growing.
- 5 23. The method of Claim 20 in which the Mycobacterium tuberculosis is continuously propagated in mid-log phase.
- 10 24. The method of Claim 20 wherein the heterologous DNA additionally comprises DNA which is not homologous to Mycobacterium tuberculosis genomic DNA.
- 15 25. A DNA construct consisting essentially of:
 - a) DNA homologous to genomic DNA of a slow-growing mycobacterium which is a genetic marker; and
 - b) DNA nonhomologous to genomic DNA of the slow-growing mycobacterium,
wherein the DNA nonhomologous to genomic DNA of the slow-growing mycobacterium is flanked by the DNA homologous to genomic DNA of a slow-growing mycobacterium.
- 20 26. The DNA construct of Claim 25 wherein the slow-growing mycobacterium is Mycobacterium bovis BCG and the genetic marker is the orotidine-5- monophosphate decarboxylase gene locus.

-50-

27. A homologously recombinant slow-growing mycobacterium having incorporated therein heterologous DNA homologous to genomic DNA of the slow-growing mycobacterium.

5 28. The homologously recombinant slow-growing mycobacterium of Claim 27, wherein the heterologous DNA is incorporated into mycobacterial genomic DNA which is a genetic marker.

10 29. The homologously recombinant slow-growing mycobacterium of Claim 28, wherein the slow-growing mycobacterium is Mycobacterium bovis BCG and the genetic marker is the orotidine-5-monophosphate decarboxylase gene locus.

15 30. The homologously recombinant slow-growing mycobacterium of Claim 29, wherein the heterologous DNA additionally comprises DNA nonhomologous to genomic DNA of the slow-growing mycobacterium.

20 31. The homologously recombinant slow-growing mycobacterium of Claim 30, wherein the DNA nonhomologous to genomic DNA of the slow-growing mycobacterium is DNA encoding a protein or polypeptide selected from the group consisting of: antigens, enzymes, cytokines, lymphokines and immunopotentiators.

-51-

32. The homologously recombinant slow-growing mycobacterium of Claim 30, wherein the nonhomologous DNA encodes an antigen of a pathogen.
- 5 33. The homologously recombinant slow-growing mycobacterium of Claim 27, wherein the heterologous DNA is not expressed and incorporation of the heterologous DNA homologous to genomic DNA of the slow-growing mycobacterium into the genomic DNA inactivates or activates a gene in the genomic DNA.
- 10 34. The homologously recombinant slow-growing mycobacterium of Claim 27 wherein the slow-growing mycobacterium is M. tuberculosis and the gene in the genomic DNA is selected from the group consisting of: the katG gene, the Hsp60 gene, aroA, lysA, uraA and M. tuberculosis DNA associated with entry into and survival inside cells of M. tuberculosis.
- 15 35. Isolated DNA of mycobacterial origin encoding orotidine-5'-monophosphate decarboxylase.
- 20 36. Isolated DNA of Claim 35 having the nucleotide sequence of SEQ ID No. 1 from nucleotide 1691 through and including nucleotide 2512.
37. Isolated orotidine-5'-monophosphate decarboxylase of mycobacterial origin.

-52-

38. Isolated orotidine-5'-monophosphate decarboxylase of Claim 37 encoded by DNA having the nucleotide sequence of SEQ ID No. 1 from nucleotide 1691 through and including nucleotide 2512.

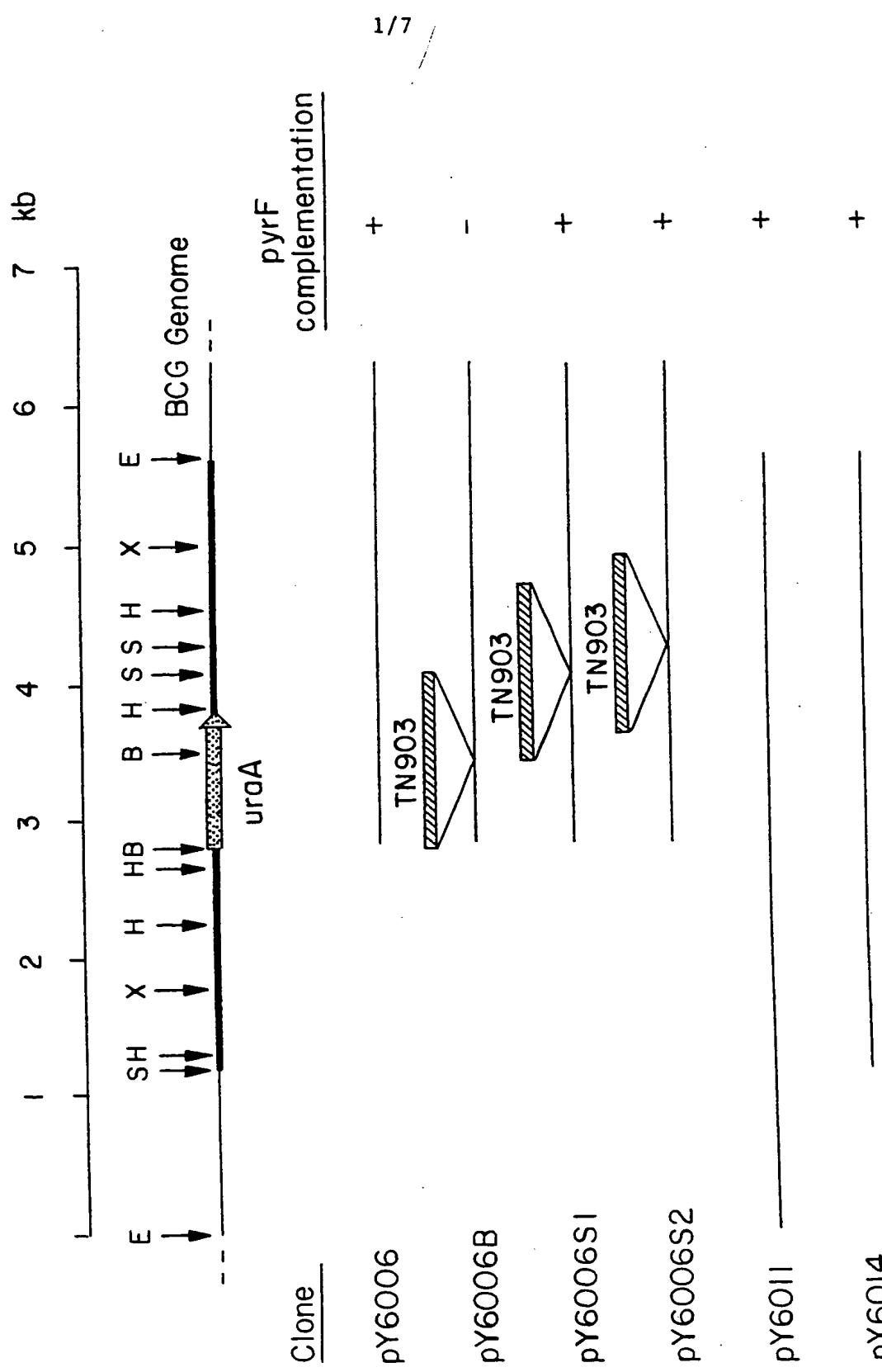


FIG. 1

GAGCTCGACCCCGCCGCCGAAACAGAGGTGGCCCCGCAGACCGAAAGGCCAAGGTGCTG	60
E L D P A A E T E V A P Q T E R P K V L	
ATCCTCGGTTCGGGGCCAATCGGATCGGCCAGGGTATCGAGTTGACTACAGCTGCGTA	120
I L G S G P N R I G Q G I E F D Y S C V	
CACCGGGCAACCACGGTGGAGCCAGGCTGGCTTGAGACCGTGTGGTCAACTGCAACCCG	180
H A A T T L S Q A G F E T V M V N C N P	
GAGACCATGGTGTCCACCGACTTCGACACCCGGACAGGGTGTACTTCGAGGCCGTTGACG	240
E T M V S T D F D T A D R L Y F E P L T	
TTCGAGGACGTCTGGAGGTCTACACGCCGAAATGGAATCCGGTAGCGGTGGCCCGGG	300
F E D V L E V Y H A E M E S G S G G P G	
GTGGCCGGCGTCATCGTCAGCTCGGCCAGACCCCGCTGGCTGCCGACCCGCTCG	360
V A G V I V Q L G G Q T P L G W R T G S	
CCGACGCCGGTCCCGCTCGTGGGACCCACCGGAGGCCATCGACCTGGCCGAGGATGCG	420
P T P G P A R G H P P E A I D L A E D A	
GCCGTTCGGCACCTGCTGAGCGAGGACTGCCGGCCAAAGTACGGCACCGCAACCACT	480
A V R R P A E R G L P A P K Y G T A T T	
TTCGCCCAGGCCGCGGATCGCCGAGGAGATCGGCTATCCGGTGTGGTGGCCGTCG	540
F A Q A R R I A E E I G Y P V L V R P S	
TATGTGCTCGGTGGTCGCGCATGGAGATCGTGTATGACGAAGAACGTTGAGGGCTAC	600
Y V L G G R G M E I V Y D E E T L Q G Y	
ATCACCCGCGCACTCAGCTATCCCCGAACACCCGGTGTGACCGCTTCCTCGAG	660
I T R A T Q L S P E H P V L V H R F L E	
GACCGGGTCGAGATCGACGTCACGCTCTGTGTGATGGCGCCGAGGTCTATATCGCCGA	720
D A V E I D V D A L C D G A E V Y I G G	
ATCATGGAGCACATCGAGGAGGCCGATCCACTCCGGTACTCGGCCTGTGCGCTGCCA	780
I M E H I E E A G I H S G D S A C A L P	
CCGGTCACGTTGGGCCGAGCGACATCGAGAAGGTGCGTAAGGCCACTGAAGCCATTGCG	840
P V T L G R S D I E K V R K A T E A I A	
CATGGCATCGCGTGGTGGGCTGCTAACGTGCAGTCGCCTCAAGGATGACGTGCTC	900
H G I G V V G L L N V Q S A L K D D V L	
TACGTCTGGAAGCCAACCGAGAGCGAGCCGTACCGTTCCGTTGTATCCAAGGCCACA	960
Y V L E A N P R A S R T V P F V S K A T	
CGGGTGCCACTCGCCAAGGCATCGCCCGGATCATGTTGGGCCACCATTGCCAGCTG	1020
A V P L A K A C A R I M L G A T I A Q L	
CCGCGGAAGGCTTGTGGCGGTACCGGGGATGGCGCCACCGCGCGCGAAACGCC	1080
R A E G L L A V T G D G A H A A R N A P	
ATCGCGGTCAACCAGGCCGTGTGCCGTTCACCGGTTCCGGCGCGACGGGGCGCC	1140
I A V N Q A V L P F H R F R R A D G A A	
ATCGACTCGCTACTCGGCCGGAGATGAAATCGACCGCGAGGTGATGGGCATCGACCGC	1200
I D S L L G P E M K S T G E V M G I D R	
GACTTCGGCAGCCGGTTCGCCAAGAGCCAGACGCCGCTACGGGTCGCTGCCGCCAG	1260
D F G S R F A K S Q T A A Y G S L P A Q	

FIGURE 2A

SUBSTITUTE SHEET (RULE 26)

GGCACAGTGTTCGTGCGTGGCAACCGGGACAAGCGGTGCTGGTGTTCGGTCAAA 1320
 G T V F V S V A N R D K R S L V F P V K
 CCGATTGGCCCACCTGGGTTTCGCGTCCTGCCACCGAAGCACCGCAGAGATCTTGCGCC 1380
 R L A H L G F R V L A T E A P Q R S C A
 GCAACGGTATTCCCTGCGACGACGTCCGAAACATTGAGCCGGCGCAGCCGGGCC 1440
 A T V F P A T T S A N I S S R R S P A A
 CCACAATGTCGGCGGTGGACGCGATCCGAGGCCGGCGAGGTCAACATGGTATCAACACTC 1500
 P Q C R R W T R S E P A R S T W
 CCTATGGCAACTCCGGTCCGCGATCGACGGCTATGAGATCCGTTGGCGGGTGGCCG 1560
 GCAACATCCCGTGCATCACACGGTGCAGGGCGATCCGCCCGTGCAGGGGATAGAGG 1620
 CCGGGATCCCGGGCGACATCGGGGTGCCTCCCTGCAGGAGCTGCACCGGGTGTGGGG 1680
 GCGTCGAGCGGTGACCGGGTTGGCTCCGGTGGCCGAGGCAAAGGCACGCCGCC 1740
 M T G F G L R L A E A K A R R G P
 GTTGTGTCTGGGCATCGATCCGCATCCCGAGCTGCTGCGGGGCTGGGATCTGGCACCAC 1800
 L C L G I D P H P E L L R G W D L A T T
 GGCGCACGGGCTGGCCCGTTCTGCGACATCTGCGTACGGGCCTTCGCTGATTCGGGT 1860
 A D G L A A F C D I C V R A F A D F A V
 GGTCAAACCGCAGGTGGCGTTTTTGAGTCATACTGGGCTGCCGGATTCCGGTGTGG 1920
 V K P Q V A F F E S Y G A A A G F A V L E
 GCGCACCATCGCGGAACCTGCGGGCCCGCAGACGTGCTGGTGTGGCCGACGCCAAGCGCGG 1980
 R T I A E L R A A D V L V L A D A K R G
 CGACATTGGGGCGACCATGTCGGCGTATGCGACGGCCTGGGTGGCGACTGCCGCTGGC 2040
 D I G A T M S A Y A T A W V G D S P L A
 CGCCGACGCCGTGACGGCCTGCCCTATTGGGCTTCGGTTCGCTGCCGGCTGCTAGA 2100
 A D A V T A S P Y L G F G S L R P L L E
 GGTGCGGGCGCCACGGCCGAGGGGTGTTGCTGCTGGCGGCCACCTCCAATCCGAGGG 2160
 V A A A H G R G V F V L A A T S N P E G
 TCGGGCGGTGAGAATGCCGCCGACGGCCGAGCGTGGCCAGTTGGTCGTGGACCA 2220
 A A V Q N A A A D G R S V A Q L V V D Q
 GGTGGGGCGCCAACGAGGCCGAGGACCCGGGATCCATGGCGTGGTGTGGCG 2280
 V G A A N E A A G P G P G S I G V V V G
 CGAACGGCGCCACAGGCCCGATCTCAGCGCCTTCACCGGGCGGTGCTGGTGGCC 2340
 A T A P Q A P D L S A F T G P V L V P G
 CGTGGGGGTGCAGGGCGGGCGCCGGAGGCCTGGCGAGGTCTTGCCTGGCGCCGGCGCATCGAG 2400
 V G V Q G G R P E A L G G L G G A A S S
 CCAGCTGTTGCCCGCGGTGGCGCGAGGTCTTGCCTGGCGCCGGCGTGGCGGATT 2460
 Q L L P A V A R E V L R A G P G V P E L
 GCGCGCCGCCGGCGAACGGATGCCGATGCCGTGCCCTATCTCGCTGCCGTAGCGGGT 2520
 R A A G E R M R D A V A Y L A A V
 GCCCTGCCACCGCGCCGCTAAATCCCACCAAGCATGGGTGGTGAGCCCAGCGCTCGTGTG 2580

FIGURE 2B

ACCAAACTCACCGCCCTGGGCCGTCGTACGCTGTGTTAACCTCTCGTTCAAATGATATT	2640
CATATTCAATAGTGGCGCTAAGTGTCCGGTTGAATCCCCGTTGAACCCCCAACAGATGGA	2700
GTCTGTGTCGTGACGTTGCGAGTCGTTCCCGAAAGCCTGGCAGGCGCCAGCGCTGCCATC	2760
GAAGCAGTGACCGCTCGCCTGGCCGCCGCCACGCCGGCGGGCCCCGTTATCGCGGCG	2820
GTCATCCCGCCTGGGTCCGACTCGGTTTGGTGTGCAACGCCGTTGAGTTAGCGTTCAC	2880
GGTAGTCAGCATGTGGCAATGGCCGCTCAGGGGTTGAGGAGCTCGGCCGCTCGGGGGTC	2940
M W Q W P L R G L R S S A A R G S	
GGGGTGGCCGAATCGGGTGCCAGTTATGCCGCTAGGATGCGCTGGCGGCGCGTACATC	3000
G W P N R V P V M P L G C A G G G V V S	
TCAGCGGTGGCTATGACCGAGCCGTGGATAGCCTCCCTCCCGAGGTGCACTCGGCAT	3060
Q R W A M T E P W I A F P P E V H S A M	
GCTGAACTACGGTGCAGGGCGTTGGGCCATGTTGATCTCCGCCACGCAGAAATGGGGAGCT	3120
L N Y G A G V G P M L I S A T Q N G E L	
CAGCGCCCAATACGAGAAGCGGCATCCGAGGTCGAGGAATTGTTGGGGTGGTGGCCTC	3180
S A Q Y A E A A S E V E E L L G V V A S	
CGAGGGATGGCAGGGGCAAGCCGCCGAGGCCTAGTCGCCGCGTACATGCCGTTCTGGC	3240
E G W Q G Q A A E A L V A A Y M P F L A	
GTGGCTGATCCAAGCCAGCGCCACTCGTGGAAATGGCCGCCAGCAACACGCCGTCA	3300
W L I Q A S A D C V E M A A Q Q H A V I	
CGAGGCCTACACTGCCCGGTAGAGCTGATGCCACTCAGGTCGAACGCCGCAACCA	3360
E A Y T A A V E L M P T Q V E L A A N Q	
AATCAAGCTCGCGGTGTTGGTAGCGACCAATTCTTGATCAACACCATTCCCATTGC	3420
I K L A V L V A T N F F G I N T I P I A	
GATCAATGAGGCCGAGTACGTGGAGATGTGGGTTCCGGCCGCCACCGATGGCGACCTA	3480
I N E A E Y V E M W V R A A T T M A T Y	
TTCAACAGTCTCCAGATCGCGCTCTCCGCGATGCCACACCAGCCCCCGCCGTGAT	3540
S T V S R S A L S A M P H T S P P P L I	
CCTGAAATCGATGAACTGCTCCCCGACACCGGGGAGGACTCCGATGAAGACGCCACAA	3600
L K S D E L L P D T G E D S D E D G H N	
CCATGGCGGTACAGTCATGGCGGTACGCCAGGATGATCGATAACTTCTTGCCGAAAT	3660
H G G H S H G G H A R M I D N F F A E I	
CCTGCGTGGCGTCAGCGCGGCCGATTGTTGGGACCCCGTCAACGGCACCCCTAACGG	3720
L R G V S A G R I V W D P V N G T L N G	
ACTCGACTACGACGATTACGTCTACCCCGTCACGCCATGGTGGCTGGCTCGAGGCCT	3780
L D Y D D Y V Y P G H A I W W L A R G L	
CGAGTTTTTCAGGATGGTGAACAATTGGCGAACTGTTGTTACCAATCGACTGGGC	3840
E F F Q D G E Q F G E L L F T N P T G A	
TTTCAGTTCTCCTCTACGTCGTTGGGATTTGCCGACGCCACATAGCCCAGATCGC	3900
F Q F L L Y V V V V D L P T H I A Q I A	

FIGURE 2C

5/7

TACCTGGCTGGGCCAGTACCCGAGTTGCTGCGCTGCCCTCACTGGCGTCATGCCCA 3960
T W L G Q Y P Q L L S A A L T G V I A H

CCTGGGAGCAATAACTGGTFTTGGCGGGCTATCCGGCCTGAGCGCCATTCCGTCTGCTGC 4020
L G A I T G L A G L S G L S A I P S A A

GATAACCGCCGTTGTACCGGAGCTGACACCCGTCGCGGCCGCCCTATGTTGGCGGT 4080
I P A V V P E L T P V A A A P P M L A V

CGCCGGGGTGGGCCCTGCAGTCGCCGCCGGCATGCTCCCCGCCCTCAGCACCCGCACC 4140
A G V G P A V A A P G M L P A S A P A P

GGCGGCAGCGGCCGCCACCGCAGCCGGCCGACGCCGCCGGCAGCTGGTTCGGAGG 4200
A A A A G A T A A G P T P P A T G F G G

GCTTCCCGCCCTACCTGGTCGGCGGTGGCGGCCAGGAATAGGTTCGGCTCGGGACAGT 4260
L P A L P G R R W R P R N R V R L G T V

CGGCCACGCCAAGGCCGCCGTCCGATTCCGCTGCAGCCGAGTCGGCGGCCAGGCCT 4320
G P R Q G R G V R F R C S R V G G P G L

CGGCGCGTGCAGGCCGTGCTGCACGGCGGGCCGCTGGCGGCCAGGCACGTGGCCA 4380
G A C A G A C C T A G P L G G K A R G H

TCGTGACGAATT
R D E F 4393

FIGURE 2D

6/7

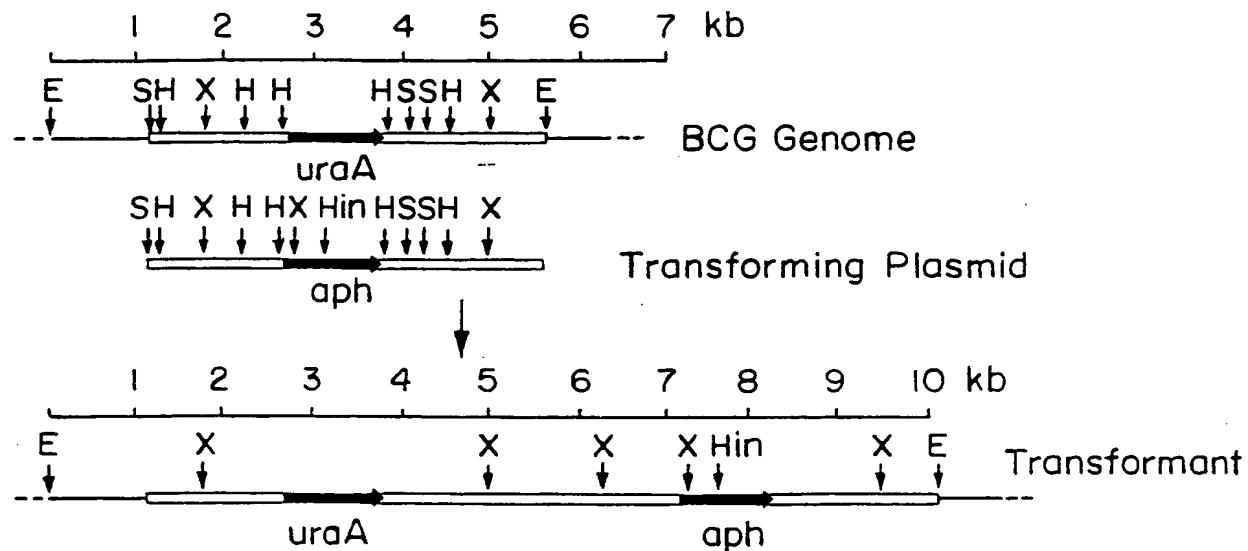


FIG. 3

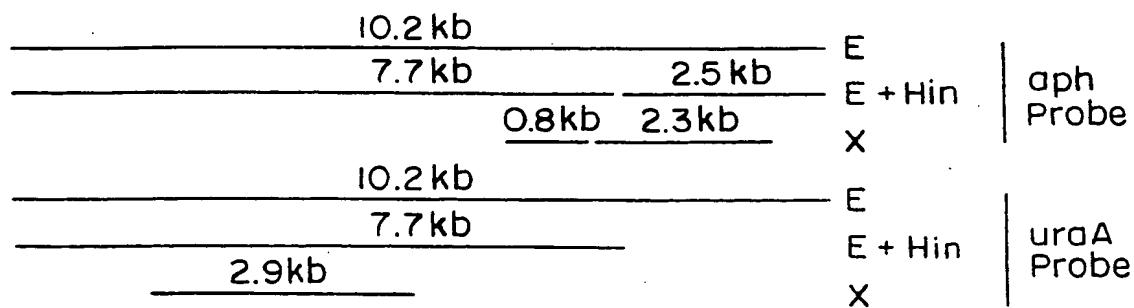
Southern Analysis

FIG. 4

7/7

FIG. 5

